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A

DISSERTATION

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DEDICATION

This Ph.D. thesis work is dedicated to

My dearest wife

Chieh (Judy) Tseng

And

Our family

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Akt (also known as protein kinase B) serves a central regulator in PI3K/Akt signaling pathways to regulate numerous physiological functions including cell proliferation, survival and metabolism. Akt activation requires the binding of Akt to phospholipid PIP3 on the plasma membrane and subsequent phosphorylation of Akt by its kinases. Growth factor-mediated membrane recruitment of Akt is a crucial step for Akt activation. However, the mechanism of Akt membrane translocation is unclear. Protein ubiquitination is a significant posttranslational modification that controls many biological functions such as protein trafficking and signaling activation. Therefore, we hypothesize that ubiquitination may be involved in Akt signaling activation.

We have demonstrated that Akt could be conjugated with non-proteolytic K63-linked ubiquitination by TRAF6 ubiquitin E3 ligase. This modification on Akt was required for membrane recruitment, phosphorylation and activation of Akt in response to growth factor stimulation. The human cancer-associated Akt E17K mutant exhibited an increase in K63-linked ubiquitination, which contributes to the enrichment of membrane recruitment and phosphorylation of Akt. Thus, we conclude that K63-linked ubiquitination is a critical step for oncogenic Akt activation and also involved in human cancer development.

Notably, the process of protein ubiquitination can be reversed by deubiquitinating enzymes (DUBs), which play a critical role to terminate signaling activation induced by ubiquitination. To further investigate how ubiquitination cycles regulate Akt activation, we have identified that CYLD as a DUB for Akt, and CYLD inhibited growth factor-induced ubiquitination and activation of Akt. Under serum-depletion condition, CYLD interacts with Akt and keep Akt under inactive state by directly removing K63-linked ubiquitination of Akt. CYLD disassociates with Akt upon growth factor stimulation, thereby allowing E3 ligases to induce ubiquitination and activation of Akt. We also demonstrated that CYLD deficiency promoted cancer cell proliferation, survival, glucose metabolism and human prostate cancer development. Therefore, we conclude that CYLD plays a critical role for negatively regulating Akt signaling activation through deubiquitination of Akt.

In summary, this study delineated the important mechanism of cycles of ubiquitination and deubiquitination of Akt in regulating membrane translocation and activation of Akt, and TRAF6 and CYLD as central switches for these processes.

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LIST OF ABBREVIATIONS

17-AAG	17-allylamino-17-demethoxygeldanamycin
ACAP1	ArfGAP with coiled-coil, ankyrin repeat and PH domains 1
BRCA1	Breast cancer type 1
c-Cbl	Casitas B-lineage lymphoma
CHIP	Carboxyl terminus of Hsc-70-interacting protein
c-IAP1/2	Cellular inhibitor of apoptosis protein 1/2
CYLD	Cylindromatosis
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ERK1	Extracellular signal-regulated protein kinase 1
FKBP51	FK506 binding protein 51
GSK3-β	Glycogen synthase kinase-β
GST	Glutathione S-transferase
HSP90	Heat-shock protein 90
HECT	Homologous to the E6-AP carboxyl terminus
HectH9	Homologous to E6AP carboxyl terminus homologous protein 9
IGF-1R	Insulin-like growth factor-1 receptor
IGFBP-5	Insulin-like growth factor-binding protein-5
IκB	Inhibitor of NF-κB

IL-1	Interleukin-1
IKKα	Inhibitor of nuclear factor kappa-B kinase subunit alpha
IRAK4	IL-1 receptor-associated kinase 4
JAMM/MPN+	JAB1/MPN/MOV34 metalloenzyme
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDM2	Murine double minute
MEF	Mouse Embryonic Fibroblast
MEKK3	Mitogen-activated protein kinase kinase kinase 3
MLK3	Mixed linage kinase 3
mTORC2	Mammalian target of rapamycin complex 2
NEDD4-1	Neural precursor cell expressed, developmentally down-regulated 4-1
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor-kappa B
OUT	Ovarian tumor-like protease
PAK1	p21 protein-activated kinase 1
PCR	Polymerase chain reaction
PDK-1	Phosphoinositide-dependent kinase-1

PH domain	Pleckstrin homology domain
PHLPP	PH domain leucine-rich repeat protein phosphatase
PI3K	Phosphatidylinositol 3-kinase
PI(3,4,5)P3	Phosphatidylinositol (3,4,5)-trisphosphate
PIN	Prostate intraepithelial neoplasia
PKB	Protein kinase B
PML	Promyelocytic leukemia protein
PP2A	Protein phosphatase 2A
PTEN	Phosphatase and tensin homolog
RANKL	Receptor activator of NF-κB ligand
RING	A really interesting new gene
RIP1	Receptor-interacting serine/threonine-protein kinase 1
RNF8	Ring finger protein 8
Skp2	S-phase kinase-associated protein 2
Smad2/3	SMAD family member 2/3
TAK1	TGF-β activating kinase-1
TGF-β	Tumor growth factor-β
TLR	Toll-like receptor
TNF	Tumor necrosis factor

TRAF6	TNF receptor-associated factor 6
TSC2	Tuberous sclerosis 2
TTC3	Tetratricopeptide repeat domain 3
UBC13/UBE2N	Ubiquitin-conjugating enzyme E2N
UBD	Ubiquitin binding domains
UCH	Ubiquitin C-terminal hydrolase
UEV1A	Ubiquitin-conjugating enzyme E2 variant 1
UIM	Ubiquitin-interacting motif
USP	Ubiquitin-specific protease

Chapter 1

Introduction

1-1. Akt/Protein Kinase B (PKB)

Akt (also known as protein kinase B) is a serine/threonine protein kinase which belongs to the AGC (cAMP-dependent, cGMP-dependent and protein kinase C) protein kinase family and shares extensive homology of kinase domain with protein kinases A, G and C (1). Akt is originally discovered as the cellular homolog of oncogene (*v-Akt*) within the mouse leukemia virus AKT8 (2). It consists of three highly homologous isoforms: Akt1, Akt2 and Akt3 (encoded by *PKBα*, *PKBβ* and *PKBγ* genes, respectively) (3). Each individual AKT isoform has been widely studied and reported to be involved in regulating different cellular processes. Through careful phenotypic analysis of distinct Akt isoform-specific knockout mice, these studies found that Akt1 may play an important role in cell survival as Akt1 knockout mice had smaller body size and $Akt1^{-/-}$ mouse embryonic fibroblasts (MEFs) exhibited greater rate of apoptosis than their wild-type counterparts (4, 5). Interestingly, Akt2 is reported to be involved in regulating glucose homeostasis as evident by aberration in glucose balance and type II diabetes-like phenotype in Akt2 knockout mice and consistently, $Akt2^{-/-}$ MEFs displayed impaired glucose utilization (6, 7). Akt3 knockout mice exhibited impaired brain and nervous system development, suggesting Akt3 may be involved in the development of nervous system (8).

1-2. The PI3K/Akt signaling pathway

Cells respond to a variety of extracellular stimuli, including growth factors or cytokines. These stimuli modulate cellular functions or homeostasis by engaging their cognate receptors and triggering respectively downstream signaling cascades. The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway transmits extracellular signals to the inside of cells (9). Also, it is well-known to play critical role in numerous biological functions such as growth, survival, migration and metabolism of the cells (1, 3, 9-11). Expectedly, deregulation of this central signaling pathway is associated with several types of human diseases, including, type II diabetes and cancers (12).

PI3K comprises the p85 regulatory subunit and p110 catalytic subunit. The p85 subunit contains two Src-homology 2 (SH2) domains and one Src-homology 3 (SH3) domain, and the p110 subunit possesses a kinase domain to phosphorylate its substrates, such as the phospholipid phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P2] (9). The PI3K/Akt pathway is activated by various growth factors, cytokines, or other stimuli through their cognate receptors (3, 9-11, 13). As shown in Figure 1-1, in response to these extracellular stimuli, PI3K phosphorylates the inositol ring of PI(4,5)P2 at the D-3 position to form PI(3,4,5) P3, which is essential for recruiting and activating Akt kinase on the plasma membrane (9). The translocation of Akt from the cytosol to the plasma membrane requires the pleckstrin homology (PH) domain at the N-terminal of Akt binding to PI(3,4,5)P3 phospholipid on the membrane (9, 11). In there, phosphoinositol dependent kinase 1 (PDK1) phosphorylates Akt at Thr^{308} within its activation loop of the kinase domain and mammalian target of rapamycin complex 2 (mTORC2) phosphorylates Akt at Ser^{473} within its hydrophobic motif of the regulatory domain respectively, resulting in full

activation of Akt kinase (11, 14). In contrast, a lipid phosphatase, phosphatase and tensin homolog (PTEN) can negatively regulate the PI3K/Akt signaling pathway by dephosphorylating PI(3,4,5)P3 at the D3 position of the inositol ring (15-17).

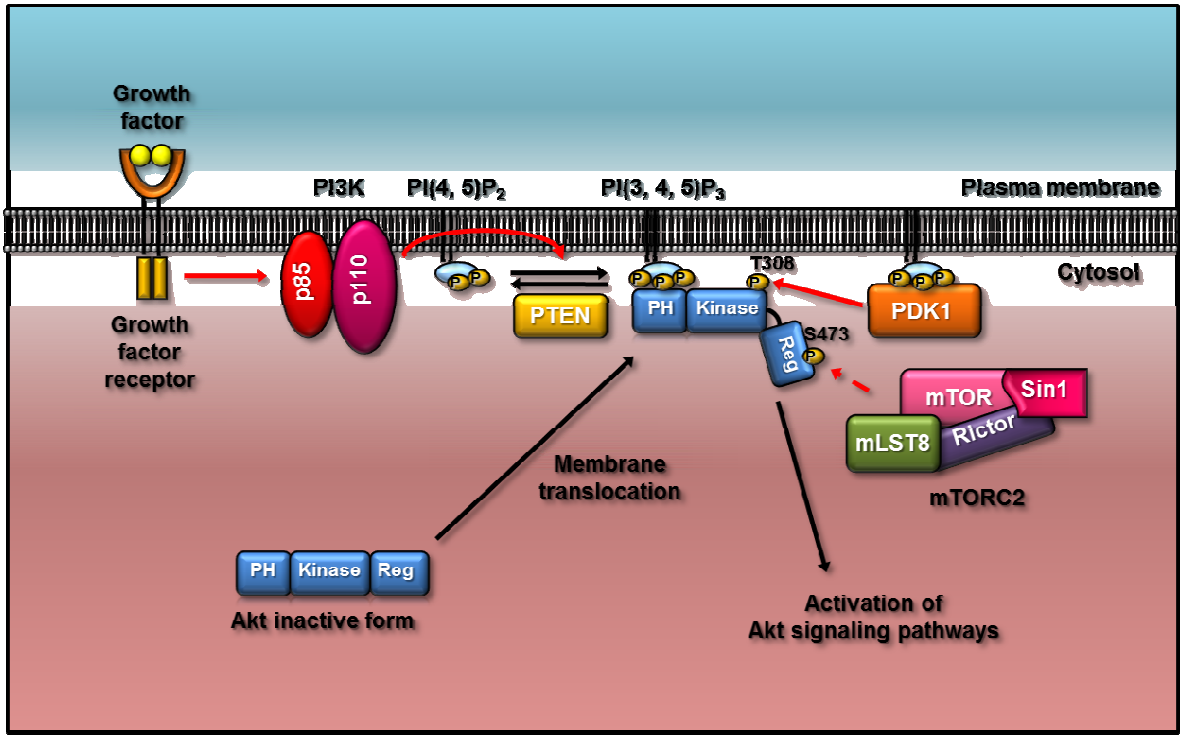

Figure 1-1. The model of growth factor-mediated phosphorylation and activation of Akt.

Growth factor receptor kinase is activated by binding to cognate growth factor. Activated growth receptor further activates PI3 Kinase. PI3 Kinase phosphorylates PIP2 to generate PIP3 phospholipid on membrane. PIP3 induces cytosolic inactive Akt translocation to the membrane and interact with PIP3 through the PH domain of Akt. Two kinases PDK1 and mTOR complex 2 phosphorylate Akt at Thr^{308} and Ser^{473} respectively, to allow full activation of Akt. Although it is well known that membrane translocation of Akt is a critical step for Akt activation, the detailed mechanism remains unclear.

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1-3. Phosphorylation cycles of Akt determines Akt kinase activity.

The activity of Akt is tightly regulated by phosphorylation and dephosphorylation cycles of Akt molecule, which serve as molecular switches to turn on/off the Akt activation. As abovementioned, full activation of Akt relies on the phosphorylation by PDK1 and mTORC2 at Thr^{308} and Ser^{473} of Akt, respectively (14, 18-20). Phosphorylation of Akt at Thr^{308} determines its kinase activity directly while phosphorylation at Ser^{473} alone does not contribute to Akt activation (21). However, dephosphorylation of Akt at both Thr^{308} and Ser^{473} causes the termination of full Akt activation, suggesting that Ser^{473} may play an important role to facilitate Akt phosphorylation at Thr^{308}.

Protein phosphatase 2A (PP2A) dephosphorylates Akt at Thr^{308}, whereas PH domain leucine-rich repeat protein phosphatase (PHLPP) dephosphorylates Akt at Ser^{473} to inhibit Akt activity (Fig. 1-2) (22-26). Notably, a higher regulatory complexity of Akt activity is mediated through Akt associating proteins, for example, dephosphorylation of Akt by PHLPP requires FK506 binding protein 51 (FKBP51) which works as a scaffold for association of Akt with PHLPP (27). Similarly, tumor suppressor promyelocytic leukemia protein (PML) has also been shown to regulate Akt activation through associating with Akt and PP2A to form a protein complex. This complex promotes PP2A-mediated dephosphorylation of Akt at Thr^{308}, suggesting that PML serves as a coordinator in the nuclear tumor suppressor network for dephosphorylation of nuclear Akt (Fig. 1-2) (28).

In response to dopamine stimulation in neurons, PP2A also interacts with another scaffold protein, β-arrestin-2, to form Akt/β-arrestin-2/PP2A signaling complex that dephosphorylates Akt (Fig. 1-2) (29). Compared with its negative role in the activation of Akt in neurons, a recent study demonstrated that β-arrestin-2 is required for

phosphorylation and activation of Akt in response to insulin stimulation in muscles (30). β-arrestin-2 recruits both Akt and Src to the activated insulin receptors, which is essential for tyrosyl phosphorylation of Akt (Fig. 1-2) (30). Several studies showed that Src-mediated tyrosyl phosphorylation of Akt at Tyr^{315} and Tyr^{326} is a prerequisite step for phosphorylation of Akt at Thr^{308} and Ser^{473} and further activation of Akt (30-34).

Importantly, deficiency of β-arrestin-2 in mice shows defect in phosphorylation of Akt and is associated with development of type 2 diabetes, whereas overexpression of β-arrestin-2 in mice enhances activation of Akt and improves the diabetes phenotype (30). This study suggests that β-arrestin-2 is an important scaffold molecule, which forms a signaling complex with Akt and Src to regulate Akt activation and insulin sensitivity (30). Therefore, these studies support the notion that β-arrestin-2 has diverse regulatory roles in the activation of Akt signaling which is dependent on different stimuli and tissue types.

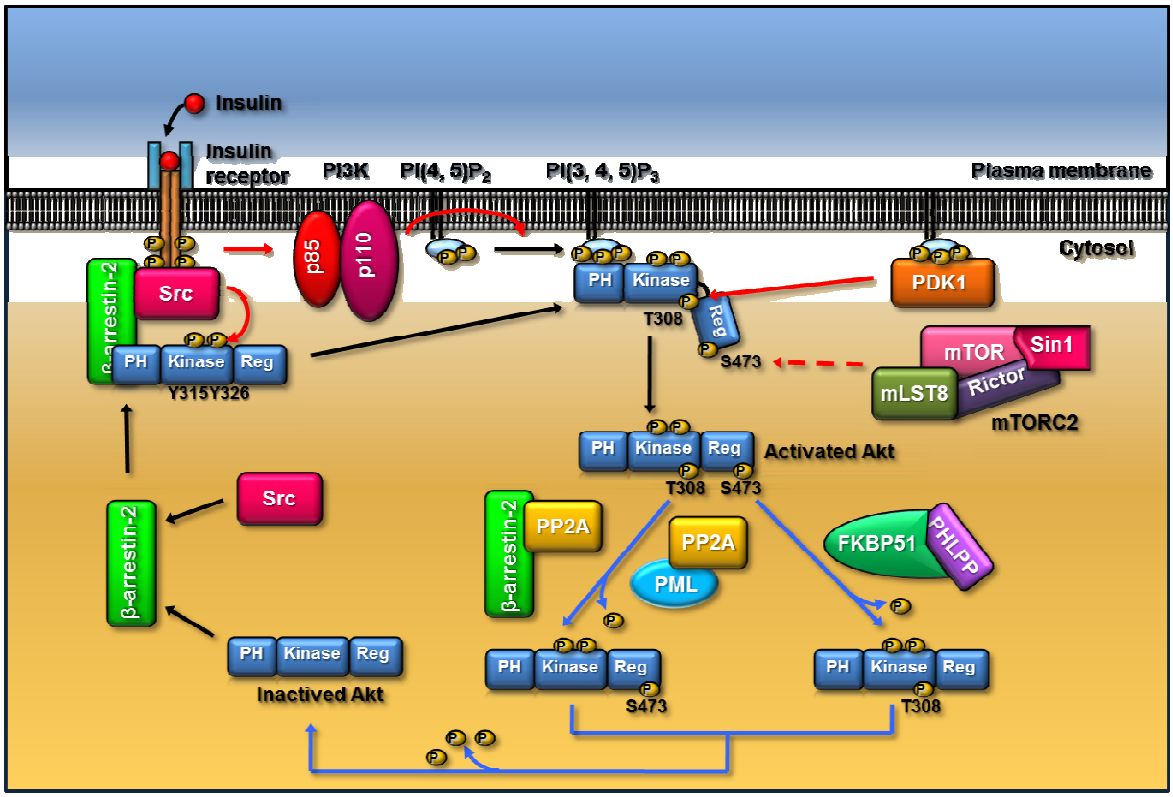

Figure 1-2. The activity of Akt is regulated by phosphorylation and dephosphorylation of Akt.

β-arrestin-2 recruits Src and Akt to the activated insulin receptor, and phosphorylation of Akt at Tyr^{315} and Tyr^{326} by Src is a prerequisite step for Akt Thr^{308} and Ser^{473} phosphorylation induced by PDK1 and mTORC2, respectively, leading to the full activation of Akt. Akt Thr^{308} dephosphorylation is triggered by PP2A phosphatase. PML or β-arrestin-2 recruits PP2A and facilitates Akt Thr^{308} dephosphorylation. Dephosphorylation of Akt at Ser^{473} is induced by PHLPP phosphates. The adaptor protein FKBP51 recruits PHLPP to elicit Akt dephosphorylation at Ser^{473}.

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1-4. Akt regulates various downstream substrates and biological functions.

Akt kinase plays a central role in the regulation of a variety of biological functions ranging from cell survival, growth, metabolism to migration by phosphorylating a broad spectrum of downstream substrates (Fig. 1-3) (1). It is well recognized that Akt inhibits cell apoptosis by promoting phosphorylation of pro-apoptotic proteins such as Bad and Foxo3a (9, 11). Furthermore, various oncogenic proteins involved in cell cycle regulation and tumorigenesis, including murine double minute (MDM2), inhibitor of nuclear factor kappa-B kinase subunit alpha (IKKα) and S-phase kinase-associated protein 2 (Skp2) E3 ligase are also phosphorylated and regulated by Akt kinase (35-41).

Additionally, Akt has been reported to phosphorylate and subsequently inactivate mTOR complex inhibitor tuberous sclerosis 2 (TSC2), resulting in activation of the mTOR signaling pathway to control cell proliferation and protein synthesis (42, 43). Importantly, Akt is also shown to regulate glucose metabolism through phosphorylating and inhibiting the activity of glycogen synthase kinase 3β (GSK3β) (44). Lastly, numerous substrates of Akt have been reported to be involved in cell migration, for example, p21 protein-activated kinase 1 (PAK1), ArfGAP with coiled-coil, ankyrin repeat and PH domains 1 (ACAP1), Skp2 and Girdin/APE are known to be phosphorylated by Akt (38, 45-47) However, further evidence is needed to determine if phosphorylation of these proteins by Akt is necessary for Akt-mediated cell migration.

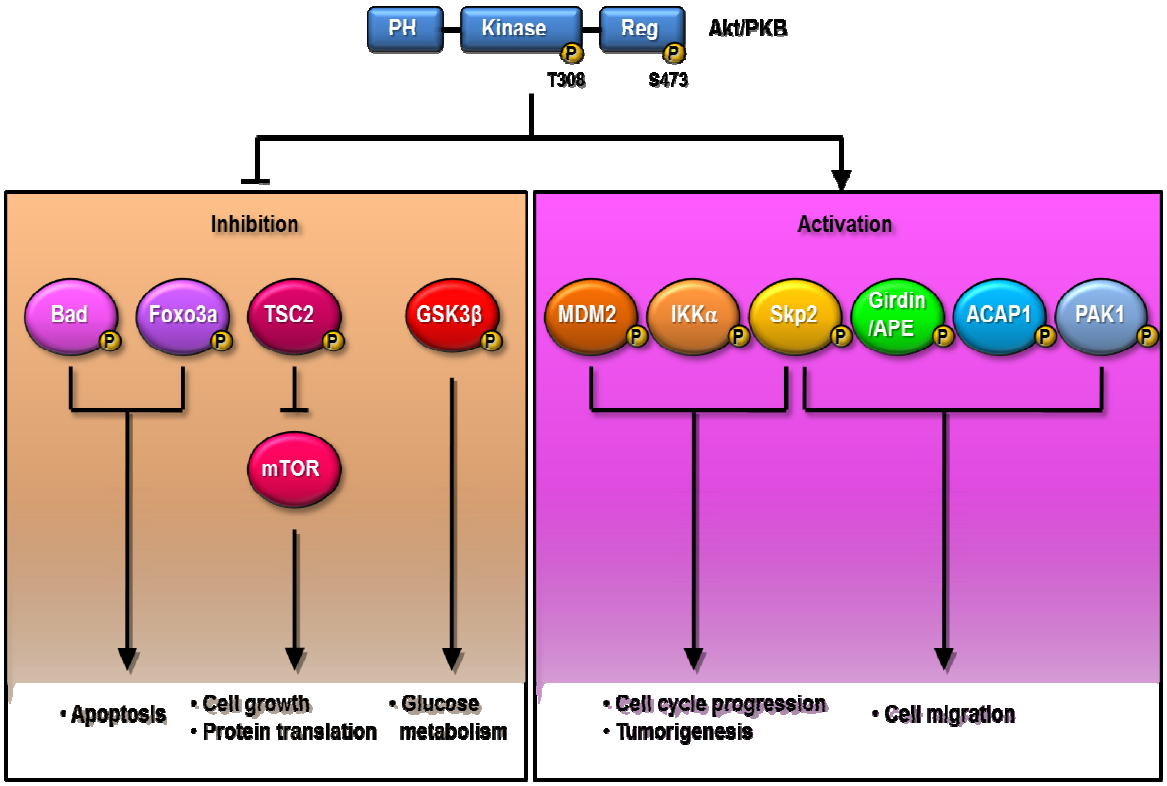

Figure 1-3. Akt regulates numerous biological functions by phosphorylating distinct protein substrates.

Akt protects cells from apoptosis by phosphorylating and inactivating proapoptotic proteins, such as Bad and Foxo3a. Akt regulates cell growth and protein translation by phosphorylating and inactivating TSC2, resulting in activation of the mTOR pathway. Akt regulates cell cycle progression and tumorigenesis by phosphorylating and activating oncogenic proteins, such as Skp2, Mdm2 and IKKα. Akt can orchestrate glucose metabolism by regulating the activity of GSK3β. Akt may also regulate cell migration by inducing the phosphorylation and activation of Skp2, Girdin/APE, ACAP1 and PAK1.

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1-5. The implication of PI3K/Akt signaling pathway in human cancer development

It has been well recognized that PI3K/Akt signaling plays a significant role in human cancer development. For example, a study in the prostate cancer model demonstrated that overexpression of insulin-like growth factor-binding protein-5 (IGFBP-5), stimulated activation of the PI3K/Akt pathway and accelerated progression of the prostate cancer from androgen-dependent to androgen-independent type (48).

Abnormal activation of Akt is detected in a variety of human cancers, and significantly, three Akt isoforms, Akt1, Akt2 and Akt3 have been reported to be overexpressed in various human cancers, such as breast cancer, liver cancer, colorectal cancer and melanoma (49-54). Recent studies reported that spontaneous Akt1 mutations associated with hyper-activation of Akt are observed in human cancers including breast cancer, ovarian cancer and colorectal cancer (55-61). The functional role of Akt in human cancer development and progression has been evaluated by several animal tumor models. For instance, *Pten*$^{+/-}$ mice with abnormal activation of Akt were found to develop multiple tumors, whereas the phenotype was reversed by deficiency of Akt1 (62, 63). Furthermore, constitutively active Akt1 specific expression in prostate of mice caused prostate intraepithelial neoplasia (PIN), a pre-malignant prostate cancer (64, 65). These studies highlight the important role of the PI3K/Akt signaling pathway in human cancer development and progression.

For intervention, several research groups have developed small molecule inhibitors to target this pathway and tested the cancer therapeutic effect of these inhibitors in clinical trials (66). However, direct targeting of this crucial signal pathway is expected to raise severe side effects such as diabetes and cardiac malfunctions (66). Therefore, a

comprehensive understanding of PI3K/Akt signaling pathway is crucial in helping us to develop innovative and effective strategies for human cancer therapies with minimum side effects. Although it is well known that the recruitment of cytosolic Akt to the plasma membrane is a critical step for Akt phosphorylation and activation (12), the underlying mechanism involved in Akt translocation to the membrane is still a mystery. It is possible that other types of post-translational modification, such as acetylation, ubiquitination, or sumoylation, may regulate the process of Akt membrane localization and activation.

1-6. The role of ubiquitination in protein degradation and other functions

Protein ubiquitination is a common post-translational modification in cell that regulates a variety of cellular functions (67-69). The function of ubiquitination is initially recognized as a signature to mark proteins for degradation through 26S proteasome-dependent manner (70). However, more and more studies demonstrate that ubiquitination is also involved in non-proteolytic functions such as receptor endocytosis, protein trafficking, DNA damage repair and activation of certain signaling pathways such as IκB kinase/nuclear transcription factor kappaB (IKK/NFκB) signaling pathway (Fig. 1-4) (12, 67, 71). These ground-breaking findings shed light on the critical role of ubiquitination in regulating numerous biological functions through distinct signal transduction pathways.

Ubiquitin is a small regulatory molecule which contains 76 amino acid polypeptides. It is highly evolutionarily conserved from prokaryote to primate (72). Ubiquitination is a covalent reaction by which ubiquitin(s) is/are attached to one or more lysine (K) residues of protein substrates (72). The process of ubiquitination involves three major steps, and each step is facilitated by distinct classes of enzymes. In the first step, ubiquitin is activated by ubiquitin activating enzyme (E1). Activated ubiquitin is transferred from E1 to ubiquitin conjugating enzyme (E2). Subsequently, ubiquitin ligase (E3) brings ubiquitin-conjugated E2 and a protein substrate together, following by the transfer of the ubiquitin from E2 to the targeted protein substrate (Fig. 1-4) (73). At present, two E1s, approximately 50 E2s and 600 E3s have been identified in human genome (67).

The fate of ubiquitinated protein is determined by types of ubiquitination on protein. Currently, three major types of ubiquitination in cells are reported, namely mono-ubiquitination, mono-ubiquitination at multiple sites and poly-ubiquitination. Mono-

ubiquitination occurs when a single ubiquitin attaches on its substrate, which is associated with protein endocytosis, endosomal sorting, DNA repair and histone regulation (74). When ubiquitination on several lysine residues of substrate takes place, it is termed multiple-ubiquitinations, which has been associated with endocytosis (74). Ubiquitin contains seven lysine residues (K6, K11, K27, K29, K33, K48 and K63), all of which may be involved in polyubiquitin chain formation *in vivo* (Fig. 1-4) (75). The function of K48-linked and K63-linked polyubiquitin chains are the best characterized at present. The K48-linked polyubiquitin chain is first identified to be recognized by the 26S proteasome for protein degradation (72). In contrast, the K63-linked polyubiquitin chain serves as a molecular platform for protein-protein interaction which is important for non-proteolytic functions including receptor endocytosis, DNA damage repair, protein trafficking and signaling activation (67-69, 76, 77). Although other types of ubiquitin modifications are also observed, the exact role they play is less understood. Recent studies suggested that they can be involved in either protein degradation or non-proteolytic functions (73, 78, 79).

Theoretically, the E2 determines the type of ubiquitination to be formed on the substrate, while the E3 determines the substrate specificity for ubiquitination (72). Most of E2 members mediate the K48-linked polyubiquitination. However, one of E2 members ubiquitin-conjugating enzyme E2N (UBC13/UBE2N), with the facilitation of its cofactor ubiquitin-conjugating enzyme E2 variant 1 (UEV1A), is a major E2 that induce K63-linked polyubiquitination (75). The E3 family members can be categorized into two major groups based on distinct ubiquitination mechanisms: [1] comprising a homologous to the E6-AP carboxyl terminus (HECT) domain; [2] consisting of a really interesting new gene (RING) or RING-like domain such as U-Box and plant homeo domain (PHD) (67). Most

of known E3 members mediate K48-linked polyubiquitination on substrates. With the exception of a few of E3 members, such as homologous to E6AP carboxyl terminus homologous protein 9 (HectH9), mouse double minute 2 (MDM2) and cellular inhibitor of apoptosis protein 1/2 (c-IAP1/2), which promote not only K48-linked polyubiquitination, but also K63-linked polyubiquitination (67, 80-83). At present, TNF receptor activating factor 6 (TRAF6) and ring finger protein 8 (RNF8) are known E3s that selectively regulate K63-linked polyubiquitination important for the innate immune response and DNA damage response, respectively (67, 81, 82).

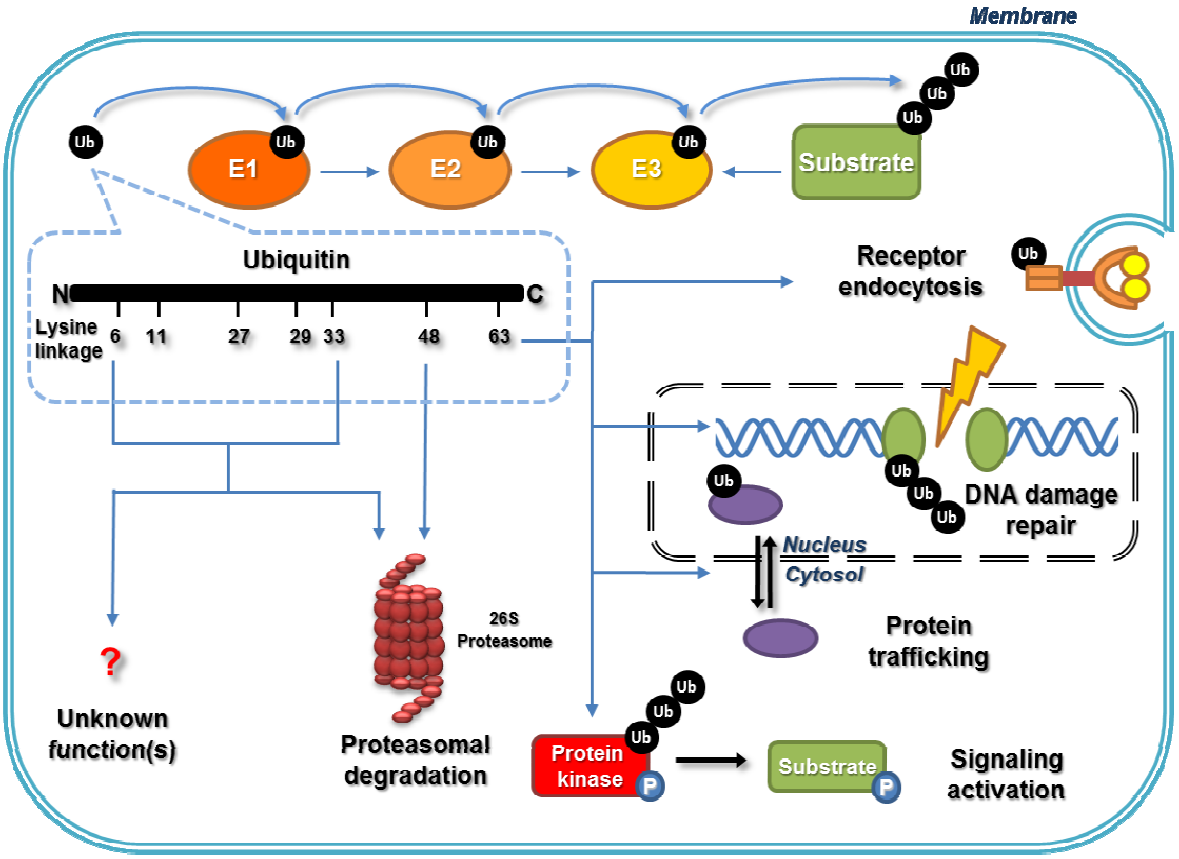

Figure 1-4. Ubiquitination regulates protein degradation or activation.

Ubiquitination reaction involves three enzymes. Ubiquitin is activated by the E1 and is subsequently transferred to the E2. The E3 provides substrate specificity and bringing the targeted substrate to the E2, resulting in protein ubiquitination. Ubiquitin consists of seven lysine residues, and the site involved in ubiquitination chains formation dictates the diverse fates of proteins. The K48-linked ubiquitination is recognized by the 26S proteasome and results in protein degradation. In contrast, the K63-linked ubiquitination regulates receptor endocytosis, DNA damage repair, protein trafficking and signaling activation. Other types of polyubiquitin chain may be involved in either protein degradation or non-proteolytic functions.

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1-7. Ubiquitination regulates degradation of Akt.

Akt activity is well-known to be modulated by phosphorylation/dephosphorylation modification. The protein stability and abundance of Akt are also critical in determining Akt activity. Akt has a relatively long half-life with its protein stability controlled by numerous stimuli (84). Heat-shock protein 90 (HSP90), a molecular chaperone, binds to Akt and maintain the stability of Akt. Cells treated with 17-allylamino-17-demethoxygeldanamycin (17-AAG), a HSP90 inhibitor, triggers Akt ubiquitination and proteasome-mediated degradation (84, 85). Following study found that carboxyl terminus of Hsc-70-interacting protein (CHIP) promotes ubiquitination of Akt and knockdown of CHIP protein expression blocks 17-AAG-mediated Akt degradation (86). This finding suggests that CHIP is essential for HSP90-mediated Akt degradation.

Furthermore, other study demonstrated that mTORC2-mediated phosphorylation of Akt at Thr^{450} is required for Akt stability. It has been shown that knockdown of mTOR complex or its component Sin1 decreases phosphorylation of Akt at Thr^{450}, followed by augmenting ubiquitination and degradation of Akt (87). Moreover, a recent study demonstrated that an E3 ligase tetratricopeptide repeat domain 3 (TTC3) is essential for ubiquitination and degradation of Akt (88, 89). In the nucleus, TTC3 showed protein-protein interaction with the active form but not inactive form of Akt, (88, 89). This finding suggested that ubiquitination of Akt by TTC3 may be a critical step to terminate Akt activity in the nucleus. Remarkably, Akt promoted phosphorylation of TTC3 at Ser^{378} and this phosphorylation is critical for enzymatic activity of TTC3 (88, 89), suggesting a possible feedback loop to negatively regulate Akt activity. In addition, Breast cancer type 1 (BRCA1) tumor suppressor is also found to associate with active Akt and to further

enhance ubiquitination and degradation of Akt (90). In summary, these recent studies suggest that CHIP, TTC3 and BRCA1 E3 ligases induce K48-linked ubiquitination of Akt to trigger Akt degradation and subsequently terminate activation of Akt signaling.

1-8. Ubiquitination regulates endocytosis and trafficking of growth factor receptors.

Ubiquitination is known to control endocytosis and membrane localization of receptor proteins, including epidermal growth factor receptor (EGFR), insulin-like growth factor-1 receptor (IGF-1R) and prolactin receptor (91-93). For example, recent study showed that in response to different concentrations of insulin-like growth factor-1 (IGF-1), IGF-1R was internalized into lipid raft compartments and the early endosome in the cell (94). The result suggested that lower concentration of IGF-1 promotes K63-linked ubiquitination of IGF-1R, whereas higher concentration of IGF-1 triggers K48-linked ubiquitination of IGF-1R (94).

MDM2 has been identified to promote K63-linked ubiquitination of IGF-1R and triggers the internalization of IGF-1R to the early endosome. In contrast, casitas B-lineage lymphoma (c-Cbl) has been found to elicit K48-linked ubiquitination of IGF-1R and initiates the internalization of IGF-1R to the lipid raft. Furthermore, neural precursor cell expressed developmentally down-regulated 4-1 (NEDD4-1) also promotes the ubiquitination of IGF-1R and controls internalization of IGF-1R to the early endosome and lipid raft (92). The results of these studies propose that distinct types of ubiquitination may control the trafficking of IGF-1R to different cellular compartments. However, it is still a puzzle if ubiquitination of IGF-1R through these E3 ligases directly controls the trafficking and signaling transduction of IGF-1R.

1-9. K63-linked ubiquitination regulates activation of protein kinases.

The activity of protein kinase is well recognized to be controlled by phosphorylation. Recently, the K63-linked ubiquitination has been found to be involved in regulating activation of some protein kinases, such as toll-like receptor (TLR) and transforming growth factor-β (TGF-β) signaling pathways (67). TGF-β activating kinase-1 (TAK1) is a central modulator that mediates activation of nuclear factor-κB (NF-κB), p38 and c-Jun N-terminal kinase (JNK) signaling pathways in response to activation of TLR and TGF-β signaling pathways (67).

More recent studies demonstrated that TAK1 can be conjugated with K63-linked ubiquitination by TRAF6 E3 ligase. Upon TGF-β signaling stimulation, TRAF6 promotes K63-linked ubiquitination of TAK1 at lysine 34 (95, 96). This type of modification is required for auto-phosphorylation and following activation of TAK1 (95). However, the mechanism underlines how ubiquitination-mediated activation of TAK1 is still unknown. The proposed models suggest two concepts: one is that K63-linked ubiquitination of TAK1 may trigger conformational change of TAK1, which then promotes auto-phosphorylation and activation of TAK1. Otherwise, K63-linked ubiquitination may work as a platform to recruit TAK1-binding protein 2 (TAB2)/TAB3 cofactors, which then initiates subsequent activation of TAK1. Indeed, a recent study supported the later concept that the ubiquitination of TAK1 functions as a scaffold to recruit mitogen-activated protein kinase kinase kinase 3 (MEKK3) to activate the mitogen-activated protein kinase (MAPK) signaling pathway (97).

Mixed linage kinase 3 (MLK3) is another example in which ubiquitination controls activation of protein kinase. MLK3 is a family member of mitogen-activated protein kinase

kinase kinase (MAP3K), which is activated by growth factors, stress and the pro-inflammatory cytokines including tumor necrosis factor (TNF) and interleukin-1 (IL-1) to activate multiple MAPK signaling pathways. One recent study demonstrated that TRAF6 promotes K63-linked ubiquitination of MLK3 and this type of modification is a crucial step for activation of MLK3 (98).

Additional studies showed that K63-linked ubiquitination of inhibitor of NF-κB kinase subunit γ (IKKγ), also known as NF-κB essential modulator (NEMO) is also required for activation of IKK complex, which is an upstream kinase that promote phosphorylation and subsequent degradation of inhibitor of NF-κB (IκB) (67, 99). The degradation of IκB separates transcription factor NF-κB from a heterodimer complex with IκB and promotes nuclear translocation and downstream gene expression of NF-κB (99). The association of the regulatory subunit IKKγ with IKKα and IKKβ is critical for activation of IKK complex although IKKγ does not have kinase activity (99). Recently, an ubiquitin-binding protein, Abin-1, has been shown to induce K27-linked ubiquitination and subsequent degradation of IKKγ, therefore, IKK complex activity and activation of NF-κB were inhibited (100). However, upon stimulation of T-cell receptor signaling, TRAF6 promoted K63-linked ubiquitination of IKKγ which is required for activation of IKK complex and NF-κB (101-103).

In summary, these above studies suggest that K63-linked ubiquitination may play a critical role for activation of a variety of protein kinases involving in several important signaling transduction pathways in the cell.

1-10. TRAF6 E3 ligase controls important signal transduction pathways.

TRAF6 is a well characterized ubiquitin E3 ligase containing a RING domain. It acts as a central mediator in regulating several signal transduction pathways, including Toll-like receptor 4 (TLR4) and interleukin-1 receptor (IL-1R) signaling pathway, to activate NF-κB signaling (67, 76, 99). Upon stimulation with ligands of TLR4/IL-1R such as lipopolysaccharide (LPS) or IL-1, TRAF6 is recruited to the receptor activated by IL-1 receptor-associated kinase 4 (IRAK4) /IRAK1/IRAK2 protein complex by promoting its oligomerization and auto-K63-linked ubiquitination (67, 76, 99). The K63-linked ubiquitination of TRAF6 has been suggested to function as a scaffold to associate with the protein kinase complex TAB2/TAB3/TAK1 (67, 99). The interaction of TAK1 with TAB2/TAB3 promotes auto-phosphorylation of TAK1, consequently activating TAK1 kinase. TAK1 then phosphorylates IKKβ to promote the activation of IKK complex including IKKα, IKKβ and the regulatory protein IKKγ (also known as NEMO) (67, 99). Active IKK complex therefore phosphorylates IκB and promotes K48-linked ubiquitination and degradation of IκB (67, 99). As a result, a subunit of NF-κB, p65, becomes free from IκB and moves into the nucleus, where it can trigger transcription of numerous target genes critical for inflammation and the innate immune response (67, 76, 99).

Studies utilizing *Traf6*$^{-/-}$ cells showed that in addition to LPS and IL-1, TRAF6 is also essential for CD40 ligand (CD40L), receptor activator of NF-κB ligand (RANKL) and tumor growth factor-β (TGF-β)-induced activation of NF-κB (Fig. 1-5) (104). TRAF6 is also involved in TGF-β-mediated phosphorylation and activation of p38 signaling pathway, but not traditional phosphorylation of SMAD family member 2/3 (Smad2/3) signaling

pathway (95, 96, 105). TGF-β induces the interaction of TRAF6 with TGF-β receptor I and further promotes ubiquitination and activation of TRAF6, in turn stimulating K63-linked ubiquitination and activation of TAK1 and subsequent phosphorylation and activation of p38 and JNK signaling pathways to regulate cell growth arrest and apoptosis (95, 96, 105). In addition, TRAF6 is shown to be essential for TGF-β-mediated epithelial-mesenchymal transition (EMT) and cell apoptosis (Fig. 1-5) (95, 96, 105). These results suggest that TRAF6 may have a dual function in TGF-β-mediated tumor suppression and cancer metastasis.

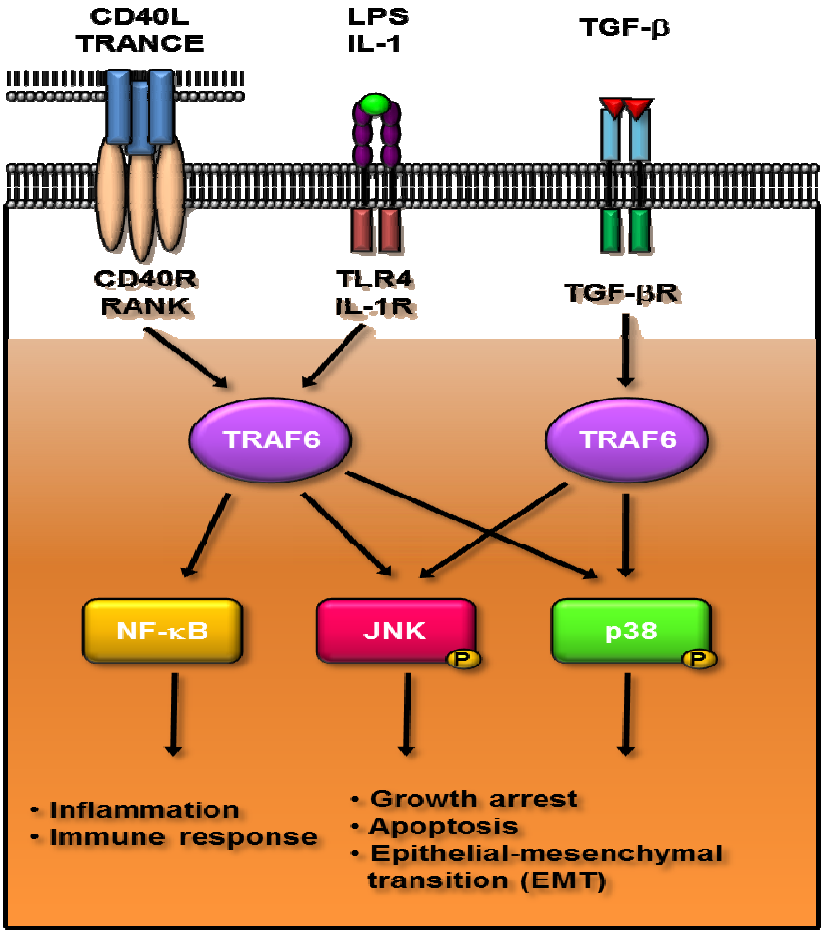

Figure 1-5. TRAF6 regulates multiple signal transduction pathways involved in the plethora of biological functions.

TRAF6 is a critical mediator for NF-κB activation upon the engagement of CD40L, TRANCE, LPS and IL-1 to their receptors, in turn regulating inflammation and innate immune response. TRAF6 is activated by TGF-β/TGF-β receptor and is required for TGF-β-mediated p38 and JNK activation. The activated p38 and JNK positively regulate cell apoptosis and EMT. Interestingly, TRAF6 is also activated upon the binding of IGF-1 to IGF-1 receptor and regulates cell survival by inducing Akt activation.

(Adapted from Cell Cycle. 1;9(3):487-9725 (2010). Reprinted with permission from Landes Bioscience.)

1-11. Ubiquitination of protein is reversible by deubiquitination.

Like other post-translational modifications, the process of ubiquitination is also reversible. Ubiquitination on protein can be removed by the deubiquitinating enzymes (DUBs), which function in opposition to the ubiquitin E3 ligases (106). One of the hallmarks for the DUBs is that the ubiquitin binding domains (UBDs) are found in most DUBs, such as the zinc-finger ubiquitin-specific protease domain (ZnF-UBP domain), the ubiquitin-interacting motif (UIM), the ubiquitin-associated domain (UBA domain) and the ubiquitin-like folds (UBL folds) (106, 107), therefore the DUBs are able to recognize and bind to the ubiquitinated protein substrates and remove ubiquitination on substrates. The function of DUB is determined by the structure of UBD, which specifically interact with the different types of ubiquitinated substrates and then triggers DUB enzyme activity to deubiquitinate substrates (107).

There are approximately 90 DUBs in human genome and they can be divided into five classes based on their sequence and structural similarity, including ubiquitin-specific protease (USP), ubiquitin C-terminal hydrolase (UCH), ovarian tumor-like protease (OTU), Josephin and JAB1/MPN/MOV34 metalloenzyme (JAMM/MPN+) (106, 107). Among them, the USP, UCH, OUT and Josephin families belong to the cysteine protease that relies on the cysteine residue as the catalytic center. Only the JAMM/MPN+ family are the zine metalloprotease which requires a metal ion, such as zinc, for its activity and catalytic mechanism (106, 107). DUBs play several important biological functions in the cell: [1] Ubiquitin precursor processing: DUBs process ubiquitin linear polyprotein precursor into free ubiquitin; [2] Ubiquitin recycling: DUBs remove K48-linked polyubiquitin chain from substrates before substrates are degraded by the 26S proteasome to release free ubiquitin;

[3] Regulation of substrate degradation: DUBs remove K48-linked polyubiquitin chain from substrates to rescue substrate degradation by 26S proteasome; [4] Regulation of substrate activity: DUBs can remove mono-ubiquitin or non-proteolytic polyubiquitin chain from substrates to terminate substrate activity; [5] Ubiquitin chain editing: DUBs edit ubiquitin chain linkage type (e.g., K48-linked to K63-linked) on the substrate by removing one ubiquitin chain linkage type before elongation of a second chain (106, 107). So far, among DUB families, the USP family members are the well-studied with known mechanisms and protein substrates characterized.

At present, the DUBs are well recognized to play significant roles in regulating critical oncogenic and tumor suppressor signal transduction pathways including PTEN, p53 and NF-κB signaling pathways (15, 67). For instance, USP7, also known as herpes virus-associated ubiquitin-specific protease (HAUSP), interacts with the ubiquitinated PTEN in the nucleus and promotes deubiquitination and subsequent nuclear export of PTEN (108). USP7 also binds to polyubiquitinated p53 and induces deubiquitination and following stabilization of p53 (109). Nevertheless, recent studies suggested that USP7 deficiency unexpectedly increases stabilization of p53 (110, 111). This phenomenon may be explicated in part by the fact that USP7 also interacts with polyubiquitinated MDM2 and increases stabilization of MDM2 (112-115). Therefore, these studies suggest that USP7 may play an oncogenic function which can facilitate human cancer development. In consistent with this concept, accumulation of USP7 protein has been found in human prostate cancer and UPS7 deficiency further inhibits cell proliferation (108).

Other USP family member, USP10, is also a DUB for deubiquitination of p53. MDM2 is formerly recognized to be an E3 ligase for ubiquitination of p53 and induces nuclear

export and degradation of p53 (116). USP10 promotes deubiquitination of p53 and reduces MDM2-indued nuclear export and degradation of p53 (117), suggesting that USP10 may have a tumor suppressive function through stabilizing p53. In line with this concept, expression of USP10 is usually deficient in renal cell carcinoma, and overexpression of USP10 inhibits normal cell transformation and tumorigenesis through regulating p53-dependent mechanism (117).

Many studies have demonstrated that USPs control ubiquitination and degradation of oncogenic proteins. For instance, USP28 interacts with Myc and leads to deubiquitination of Myc and further preventing Myc from degradation (118, 119). Notably, overexpression of USP28 has been found in several human cancers, and USP28 deficiency suppresses cell proliferation and transformation (119), suggesting that USP28 may have an oncogenic function. In addition, one recent study showed that USP2 is a DUB for deubiquitination of cyclin D1 and MDM2. USP2 interacts with cyclin D1 and MDM2 and induces deubiquitination and stabilization of cyclin D1 and MDM2, and USP2 deficiency leads to reduced protein expression of cyclin D1 and MDM2, resulting in suppression of cell proliferation (120, 121).

1-12. The role of DUBs in regulating NF-κB signaling pathways

Cylindromatosis (CYLD) and A20 proteins are well-known DUBs that play negative regulatory roles in NF-κB signaling activation by promoting deubiquitination of critical modulators such as TRAF6 and IKKγ (122, 123). CYLD has been found to be frequently mutated in patients associated with some inherited diseases including familial cylindromatosis, which is characterized by development of multiple benign tumors from skin appendages (cylindromatosis) (122, 124). CYLD exhibits specific deubiquitinase activity toward K63-linked ubiquitination of critical modulators in NF-κB signaling activation. These include TRAF2, TRAF6 and NEMO to decrease their activities in regulating activation of NF-κB (67, 122, 125-128). In addition, CYLD also inhibits activation of TAK1 and IKK complex through cleaving free unanchored K63-linked ubiquitination chains (129). Notably, analyses of the genetically engineered mouse model of CYLD has corroborated that CYLD has tumor suppressive functions *in vivo*. Several studies demonstrated that *Cyld*-null mice are highly sensitive to chemical-induced tumorigenesis such as skin cancers, colitis and colon cancers (130-133).

CYLD and an E3 ligase ITCH form a protein complex, which removes K63-linked ubiquitination chain on TAK1 by CYLD and subsequently promotes K48-linked ubiquitination chain on TAK1 by ITCH, leading to inactivation and degradation of TAK1 and subsequently terminates NF-κB activation (134). This study suggests that different types of polyubiquitination chains such as K63-linked and K48-linked ubiquitination chains can be distinctively exploited to regulate activation and inactivation of protein kinase.

A20 belongs to the ovarian tumor-like protease (OUT) deubiquitinase family. It includes an N-terminal OTU-type DUB domain and seven C-terminal zinc fingers (123). A20 was originally identified as a negative regulator of NF-κB signaling induced by innate immune signals such as tumor necrosis factor (TNF) and toll-like receptors (135). A20 can cleave K63-linked ubiquitination on TRAF6, receptor-interacting serine/threonine-protein kinase 1 (RIP1), RIP2 and IKKγ/NEMO to terminate NF-κB signaling activation (123). However, the unique feature of this DUB is that it also has E3 ubiquitin ligase activity to perform ubiquitin chain editing on substrates through replacing the K63-linked ubiquitination chain with proteasome-targeting K48-linked ubiquitination chain (136). In recent years, several lines of evidence supported the correlation between A20 and human cancers. For instance, one study reported that a dramatic loss of A20 expression is found in cases of non-Hodgkin's lymphoma, Burkett's lymphoma and T-cell lymphomas (137). Another study also demonstrated that inactivating mutations in A20 are found in large numbers of cases of marginal zone lymphoma (138). Taken together, these data suggest that A20 is a potential tumor suppressor in human lymphoma by regulating activation of NF-κB signaling.

Chapter 2

Materials and Methods

2-1. Cell lines and culture

MEFs from *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ *and Cyld*$^{+/+}$ *and Cyld*$^{-/-}$ mice were prepared as previously described (104, 139). MEFs, NIH3T3, COS-1, PC-3, DU-145, MDA-MB-231 and 293T cells were purchased from the American Type Culture Collection and cultured in DMEM containing 10% FBS with and penicillin/streptomycin in 5% CO2 at 37°C.

2-2. Antibodies and reagents

Antibodies against TRAF6, ubiquitin and N-cadherin from Santa Cruz Biotechnology; antibodies against pan-Akt isoforms, phospho-Akt (Thr^{308}), phospho-Akt (Ser^{473}), cleaved caspase-3, phospho-Foxo1 (Thr^{24})/Foxo3a (Thr^{32}), ERK, phospho-ERK, TSC2, phospho-TSC2 (Ser^{1462}), phospho-GSK3β (S9) and CYLD from Cell Signaling; antibodies against α-tubulin, β-actin and Flag-tag (M2) from Sigma; antibody against Glut1 from Abcam; antibody against Glut4 from Millpore; antibody against GSK3β from BD Transduction Lab; antibody against Xpress-tag from Invitrogen; antibody against HA-tag from Covance.

Reagents were purchased from the following sources: Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) from HyClone; complete protease inhibitor cocktail from Roche; human recombinant IGF-1 , EGF and N-Ethylmaleimide from Calbiochem; human recombinant IL-1β from Peprotech; LPS from Sigma; doxorubicin from MP Biomedicals; cisplatin from ALEXIS Biochemicals; LY294002 and wortmannin from Cell Signaling; lipofectamine 2000 from Invitrogen.

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2-3. Plasmid DNAs

Flag-TRAF6, Flag-TRAF6 C70A, pGEX-4X1-TRAF6, and pGEX-4X1-TRAF6 C70A constructs have been described previously (140). Akt KD (Akt K179A) and Mdm2 have been described previously (39). $(His)_6$-ubiqutin and HA-Akt1 were gifts from Drs. D. Bohmann and M.C. Hung, respectively. Flag-Smurf2 and Flag-FBW7 were obtained from Drs. J.L. Wrana and M.H. Lee, respectively. Flag-c-IAP1 and Flag-c-IAP2 were obtained from Dr. X. Yang. Myc-ITCH and HA-Cbl-b constructs were from

Drs. A.M. Weissman and S. Lipkowitz, respectively. HectH9, $(His)_6$-ubiqutin K6R, $(His)_6$-ubiqutin K11R, $(His)_6$-ubiqutin K48R, and $(His)_6$-ubiqutin K63R were from Dr. M. Eilers. Flag-HA-USP1, Flag-HA-USP3, Flag-HA-USP5, Flag-HA-USP8, Flag-HA-USP10, Flag-HA-USP21, Flag-HA-USP26, were described previously (121). Myc-USP7 was obtained from Dr. Pier Paolo Pandolfi (108). Flag-USP22 was obtained from Dr. Didier Devys. Flag-CYLD and Flag-CYLD-C/A (C601A) were obtained from Dr. Xin. Lin.

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2-4. Sited- directed mutagenesis of Akt

pcDNA6-HA-Akt1 K8R, pcDNA6-HA-Akt1 K14R, pcDNA6-HA-Akt1 K20R, pcDNA6-HAAkt1 K30R, pcDNA6-HA-Akt1 K39R, pcDNA6-HA-Akt1 K64R, pcDNA6-HA-Akt1 E17K, and pcDNA6-HA-Akt1 K8R/E17K constructs were generated using a site-directed mutagenesis kit (Stratagene) according to the manufacturer's standard procedures with pcDNA6-HA-Akt1 as the template.

2-5. Immunoprecipitation (IP), immunoblotting (IB) and immunofluorescence (IF) assays

IP, IB and IF were performed essentially as described elsewhere (39, 139). For protein-protein interactions, cells were lysed by E1A lysis buffer [250 mM NaCl, 50 mM HEPES (pH 7.5), 0.1% NP- 40, 5 mM EDTA, protease inhibitor cocktail (Roche)].

2-6. GST pull-down assay

For *in vitro* TRAF6 and Akt1 interaction, GST, GST-TRAF6, and GSTTRAF6 C70A proteins purified from the bacterial lysates of BL21

competent cells transformed with pGEX-4X1, pGEX-4X1-TRAF6, and pGEX-4X1-TRAF6 C70A using glutathione-agarose beads according to the manufacturer's standard procedures. The GST, GST-TRAF6, and GST-TRAF6 C70A proteins bound to glutathione Sepharose beads (Amersham Biosciences) were then incubated with the in vitro translated [35S]-Akt1 for 4 h at 4°C in the interaction buffer [20 mM HEPES (pH 7.9), 150 mM KCl, 5 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.1% (v/v) NP-40, 0.1% (w/v) BSA, 1 mM PMSF, and 10% glycerol], washed with NETN buffer [20 mM Tris (pH 8.0), 100 mM NaCl, 6 mM MgCl2, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM DTT, 8% glycerol, and 1 mM PMSF] 4 times, and subjected to 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography.

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2-7. *in vivo* and *in vitro* ubiquitination assay

In vivo ubiquitination assays were performed as described elsewhere (141). In brief, 293T cells were transfected with the indicated plasmids for 48 h and lysed by denatured buffer (6 M guanidine-HCl, 0.1 M Na2HPO4/NaH2PO4, 10 mM imidazole), followed by nickel bead purification and Immunoblot analysis. In vitro ubiquitination assays using GST-TRAF6 and GSTAKT- Flag were performed as described elsewhere (140). Purified GST-TRAF6 and GST-AKT-Flag were incubated for 3 h at 37°C in 20 μl of reaction buffer [20 mM Hepes (pH 7.4), 10 mM MgCl2, 1 mM DTT, 59 μM ubiquitin, 50 nM E1, 850 nM of Ubc13/UeV1a, 1 mM ATP, 30 μM creatine phosphate, and 1 U of creatine kinase]. After incubation, the beads were washed five times in Buffer A [20 mM Tris (pH 7.4), 250 mM NaCl, 1 mM DTT, 1 mM sodium orthovanadate, 2 mM EDTA, and 1% Triton X-100] and two times in low-salt buffer [20 mM Tris [pH 7.4], 25 mM NaCl, and 1 mM DTT]. Beads were then resuspended in 1% SDS (in water) and boiled for 10 min. Dissociated proteins were diluted with 2×600 μl Buffer A, and the supernatant fluid was precleared with Protein A/G beads for 1 h, and immunoprecipitated overnight with 1 μg anti-Flag antibody, after which Protein A/G beads were added for an additional 1 h. Beads were washed 4 times with Buffer A and 2 times with low-salt buffer. Proteins were eluted in SDS-sample buffer, subjected to SDSPAGE, transferred to nitrocelluose membrane, and immunoblotted with anti-ubiquitin.

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2-8. Endogenous ubiquitination assay

Cells were seeded in 100 mm^2 dishes and were serum-starved for 24 hours in DMEM containing 0.1% FBS. After starvation, cells were treated with/without 50 ng/ml IGF-1 for 15 min. Cells were lysed by RIPA lysis buffer [50 mM TrisHCl pH7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS and protease inhibitor cocktail (Roche)] with 10 mM N-Ethylmaleimide (Calbiochem). Immunoprecipitation was performed to pull-down polyubiquitinated Akt proteins. Samples were subjected to SDS-PAGE and immunoblotted with anti-ubiquitin antibody.

2-9. *in vitro* deubiquitination assay

To purify ubiquitinated Akt (Akt-Ub), HEK293T were transfected with HA-Akt along with His-Ub. After 48 hours, the cells were lysed with RIPA buffer (1% NP-40) and immunoprecipitated by anti-HA antibody. Purified Akt-Ub was washed by RIPA buffer for 4 times. To purify CYLD deubiquitinase, HEK293T were transfected with Flag-CYLD-WT or Flag-CYLD-C/A for 48 hours and lysed with Flag lysis buffer. The cell extracts were subjected to immunoprecipitation using anti-Flag antibody, followed by Flag peptide elution. To perform *in vitro* ubiquitination assays, the purified Akt-Ub was incubated with each purified CYLD-WT or CYLD-C/A in a deubiquitination buffer (50 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA, 10 mM DTT and 5% glycerol) for 2 hours at 37°C. After incubation, proteins on the beads were eluted in SDS-sample buffer, subjected to SDS-PAGE, transferred to nitrocelluose membrane, and immunoblotted with anti-Akt antibody.

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2-10. Retroviral/lentiviral production and infection

For TRAF6 overexpression by retroviral infection, retroviral plasmid PMX, PMX-TRAF6, and PMX-TRAF6 C70A were transfected into

Phoenix packing cells for 2 days, and the virus-containing medium was harvested and used to infect primary *Traf6*$^{+/+}$ *and Traf6*$^{-/-}$ MEFs.

For TRAF6 expression knockdown by lentiviral short hairpin RNA (shRNA) infection, 293T cells were cotransfected TRAF6 or GFP control shRNA with packing plasmids (deltaVPR8.9) and envelope plasmid (VSV-G) using Lipofectamine 2000 reagent according to the manufacturer's instructions. TRAF6-lentivivral shRNA-1 (5'-GCCACGGGAAATATGTAATATCT-3'), TRAF6-lentivivral shRNA-2 (5' CGAAGAGATAATGGATGCCAAC-3'), control shRNA (5'-GCAAGCTGACCCTGAAGTTC-3') were transfected with packing plasmids into 293T cells for 2 days, and virus particles containing TRAF6 or control shRNAs were used to infect PC-3 cells. All the infected cells were cultured in medium containing 2 μg/ml puromycin for 4 days.

For CYLD expression knockdown by lentiviral short hairpin RNA (shRNA) infection, HEK293T cells were cotransfected control or CYLD shRNA with packing plasmids (deltaVPR8.9) and envelope plasmid (VSV-G) by using calcium phosphate precipitation technique. CYLD-lentivivral shRNA #1 (5'- TACTTAGACTCAACCTTATTC-3'), CYLD-lentivivral shRNA #2 (5' AAGAAGGTCGTGGTCAAGGTC-3'), control shRNA (5'-GCAAGCTGACCCTGAAGTTC-3') were transfected with packing plasmids into HEK293T cells for 2 days, and virus particles containing CYLD or control shRNAs were used to infect PC-3, DU-145 and MDA-MB-231 cells. All the infected cells were cultured in medium containing 2 μg/ml puromycin for selection up to 1 week.

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2-11. PIP3 phospholipid binding

293T cells transfected with WT HA-Akt and various HA-Akt mutants for 48 h were lysed by E1A buffer, and the cell lysates were incubated with control beads or PIP3 beads (Enchelon) overnight. The beads were washed four times with E1A buffer and subjected to Immunoblot analysis.

2-12. Cell membrane fractionation assay

Cells were seeded in 100 mm^2 dishes and were serum-starved for 24 hours in DMEM containing 0.1% FBS. After starvation, cells were treated with 50 ng/ml IGF-1 in indicated times. Cytosolic and membrane fractions were prepared using the ProteoExtract kit (Calbiochem) according to the manufacturers' standard procedures.

2-13. Cell growth assay

Cyld$^{+/+}$ and *Cyld*$^{-/-}$ MEFs (5× 10^3 cells), DU-145 or MDA-MB-231 cells with control and CYLD knockdown (5× 10^3 cells), or PC-3 cells with control and CYLD knockdown (2× 10^3 cells) were seeded in 12 wells plate in triplicate. Cells were harvested, stained with trypan blue and counted viable cells every two days up to six days. Viable cells were counted on hemocytometer directly under the phage-contrast microscope.

2-14. Cell apoptosis assay

Cell were seeded in 60 mm^2 dishes in triplicate and were either serum-starved (0.1% FBS) or cultured in 10% FBS for 2 days or treated with/without 50 μM Cisplatin for 24 hours. Cells were collected and labeled with Annexin V-FITC apoptosis detection kit (BD Pharmingen) according to the manufacturers' standard procedures, followed by flow cytometry analysis.

2-15. Glucose uptake assay

Cells were seeded in 60 mm^2 dishes in triplicate. After 24 hours, cells were refreshed with 0.1% FBS and glucose-free DMEM for 24 hours serum-starvation. Cells treated

with/without 50 ng/ml IGF-1 were grown in the presence of fluorescent glucose analog 2-NBDG (50 μM; Invitrogen) for 1 hour (for PC-3 cells) and 24 hours (for MEFs), respectively. 2-NBDG uptake ratio by cells was analyzed by FACS analysis.

2-16. *in vivo* tumorigenesis assay

PC-3 stable cells with control and TRAF6 or CYLD knockdown (2×10^6 cells) or DU-145 stable cells with control and CYLD knockdown (5×10^6 cells) mixed with matrigel (1:1) were subcutaneously injected into the left flank of 6-week-old athymic nude mice. Tumor sizes of mice were measured weekly by using a caliper, and tumor volume was determined by using the standard formula: $L\times W^2\times0.52$, where L is the longest diameter and W is the shortest diameter.

2-17. Assessing Akt kinase activity *in vivo* in mice tissues

Heart and skeletal tissues isolated from 2-week-old *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ mice (n=5) were lysed in RIPA buffer, and lysates were used to determine Akt activity using the Akt kinase assay kit (Cell Signaling) according to the manufacturer's standard procedures. For IGF-1 injection, 2 week-old *Traf6*$^{+/+}$ *and Traf6*$^{-/-}$ mice (n=4) were starved for 6 h and intraperitoneally injected with IGF-1 (0.5 mg/kg) for various times. Skeletal and heart muscles were then isolated for the in vitro Akt kinase assay.

Chapter 3

The E3 ligase TRAF6 regulates Akt ubiquitination and activation

Most of this work has been published in:

W-L Yang, J. Wang, C-H Chan, S-W Lee, A. D. Campos, B. Lamothe, L. Hur, B. C. Grabiner, X. Lin, B. G. Darnay, H-K Lin. **The E3 ligase TRAF6 regulates Akt ubiquitination and activation.** Science 325 (5944): 1134-1138 (2009)

RATIONALE

Protein ubiquitination is a significant posttranslational modification that controls a variety of biological functions (72, 142). Although ubiquitination often causes protein degradation, a certain type of ubiquitination which is essential for non-proteolytic functions has been discovered recently (72, 142). Ubiquitination through Lys^{48} (K48)-linked ubiquitin chain normally promotes proteins for degradation, whereas ubiquitination through Lys^{63} (K63)-linked ubiquitin chain has a crucial role in protein trafficking, DNA damage response and signaling activation (72, 142). Akt plays a critical central modulator of cell signaling pathways that regulate cell growth, cell survival, cell cycle progression and cellular metabolism (1, 9). Current model suggests that membrane translocation of Akt by growth-factor stimuli is a crucial step for Akt signaling activation. However, it is not clear how Akt is recruited to the plasma membrane. Since some of important functions of ubiquitination are to promote protein trafficking and signaling activation, in this study, I hypothesized that Akt may undergo non-proteolytic ubiquitination and this type of modification may facilitate member translocation of Akt to further promote phosphorylation and activation of Akt.

RESULTS

3-1. Akt is undergoing K63-linked ubiquitination and TRAF6 is E3 ligase for Akt ubiquitination.

To test our hypothesis, first of all we determined whether Akt is ubiquitinated in cells. We found that Akt was ubiquitinated in the absence of proteasome inhibitor MG132, suggesting ubiquitination of Akt is not responsible for protein degradation (Fig. 3-1A). We also demonstrated that ubiquitination occurred on Akt through K63-linked but not through K48-linked polyubiquitination (Fig. 3-1B). In addition, we screened a panel of ubiquitin E3 ligases for ubiquitination of Akt. Although Mdm2 E3 ligase associates with Akt (40, 41, 139), it failed to promote ubiquitination of Akt (Fig. 3-1C). Other E3 ligases which promote K48-linked ubiquitination on the substrate, including c-IAP1, c-IAP2, Cbl-b, Itch, Smurf2, and Fbw7, also failed to induce Akt ubiquitination (Fig. 3-1C). Interestingly, overexpression of c-IAP1 and c-IAP2 reduced rather than promoted ubiquitination of Akt (Fig. 3-1C).

Because our preliminary results shown that Akt underwent K63-linked ubiquitination, we focused on TRAF6 and HectH9, two ubiquitin E3 ligases that catalyze K63-linked ubiquitination of the substrate. TRAF6 has an important regulatory role in TLR/IL1-R signal transduction pathway, and HectH9 is involved in the oncogenic activation of Myc, respectively (80, 99, 140). Our results showed that TRAF6 but not HectH9 promoted ubiquitination of Akt (Fig. 3-1D).

We tested whether kinase activity of Akt is required for ubiquitination of Akt by TRAF6 by using its constitutively activated (Akt-CA) and Akt kinase-dead (Akt-KD) mutants. The result showed that both Akt mutants can be ubiquitination by TRAF6,

suggesting activity of Akt was not required for TRAF6-mediated ubiquitination (Fig. 3-1E). Next, we determined whether TRAF6 E3 ligase activity is required for ubiquitination of Akt. We found that the TRAF6 $Cys^{70}\rightarrow Ala^{70}$ [C70A] mutant, which loses E3 ligase activity (140), had compromised activity for ubiquitination of Akt (Fig. 3-1F). However, this effect was not due to deficiency of interaction between TRAF6 mutant and Akt (Fig. 3-1G). Although TRAF6 promoted ubiquitination of Akt, it did not decrease the abundance of Akt in cells (Fig. 3-1D and 3-1F), suggesting that TRAF6 does not mediate K48-linked ubiquitination on Akt. Certainly, TRAF6 induced K63-linked but not K48-linked ubiquitination of Akt (Fig. 3-1H). To further confirm previous results, we conducted *in vitro* ubiquitination assay of Akt. The result showed that TRAF6, but not its C70A mutant, induced Akt ubiquitination *in vitro* (Fig. 3-1I). Therefore, these results suggest that TRAF6 is an E3 ubiquitin ligase of Akt.

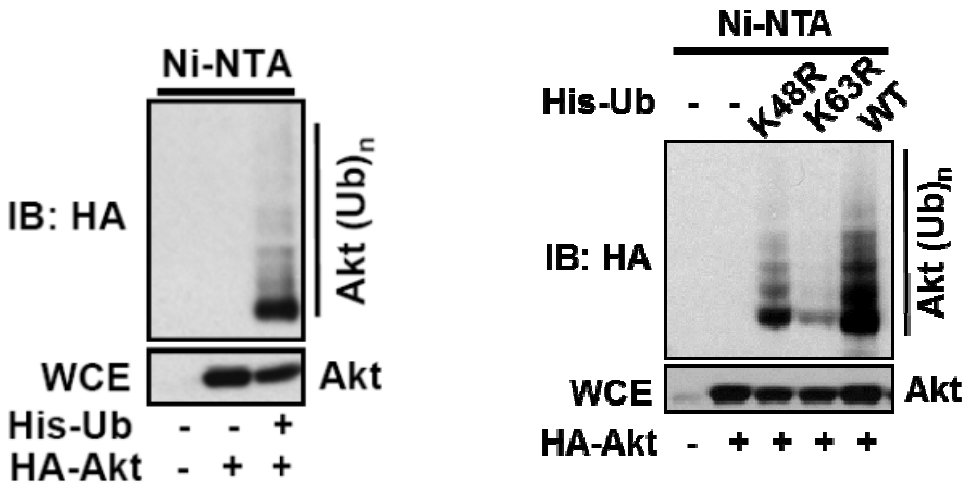

Figure 3-1. TRAF6 is an E3 ubiquitin ligase for Akt.

(A) Immunoblot (IB) of lysed 293T cells, transfected with hemagglutinin (HA)-Akt and His-ubiquitin (His-Ub) constructs for 48 h. Ni–nitrilotriacetic acid (NTA) indicates nickel bead precipitate.

(B) IB of lysed 293T cells transfected with Akt along with His-Ub-WT, His–Ub K48R, or His–Ub K63R constructs. WT indicates wild type; WCE, whole-cell extracts.

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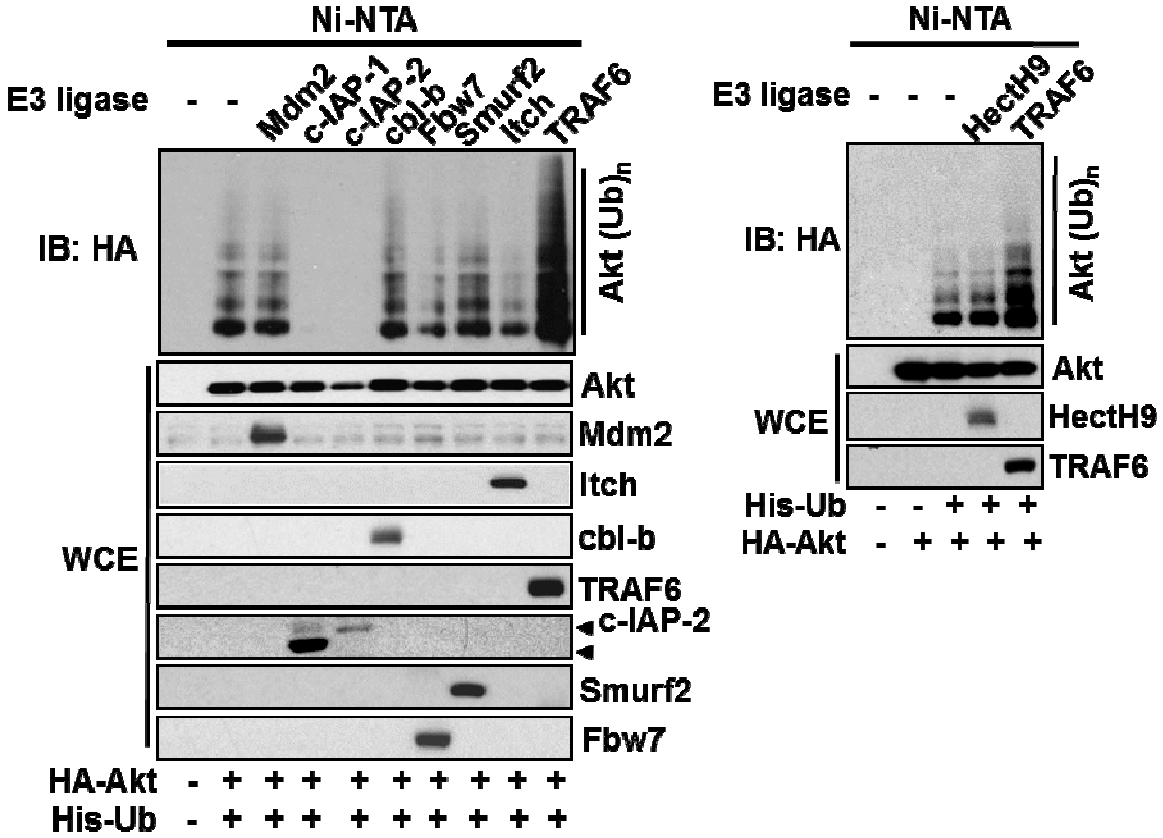

Figure 3-1. TRAF6 is an E3 ubiquitin ligase for Akt.

(C) IB of lysed 293T cells transfected with HA-Akt, His-Ub, along with various E3 ligases for Akt E3 ligase candidate screening.

(D) IB of lysed 293T cells transfected with HA-Akt and His-Ub, along with HectH9 or TRAF6 E3 ligases.

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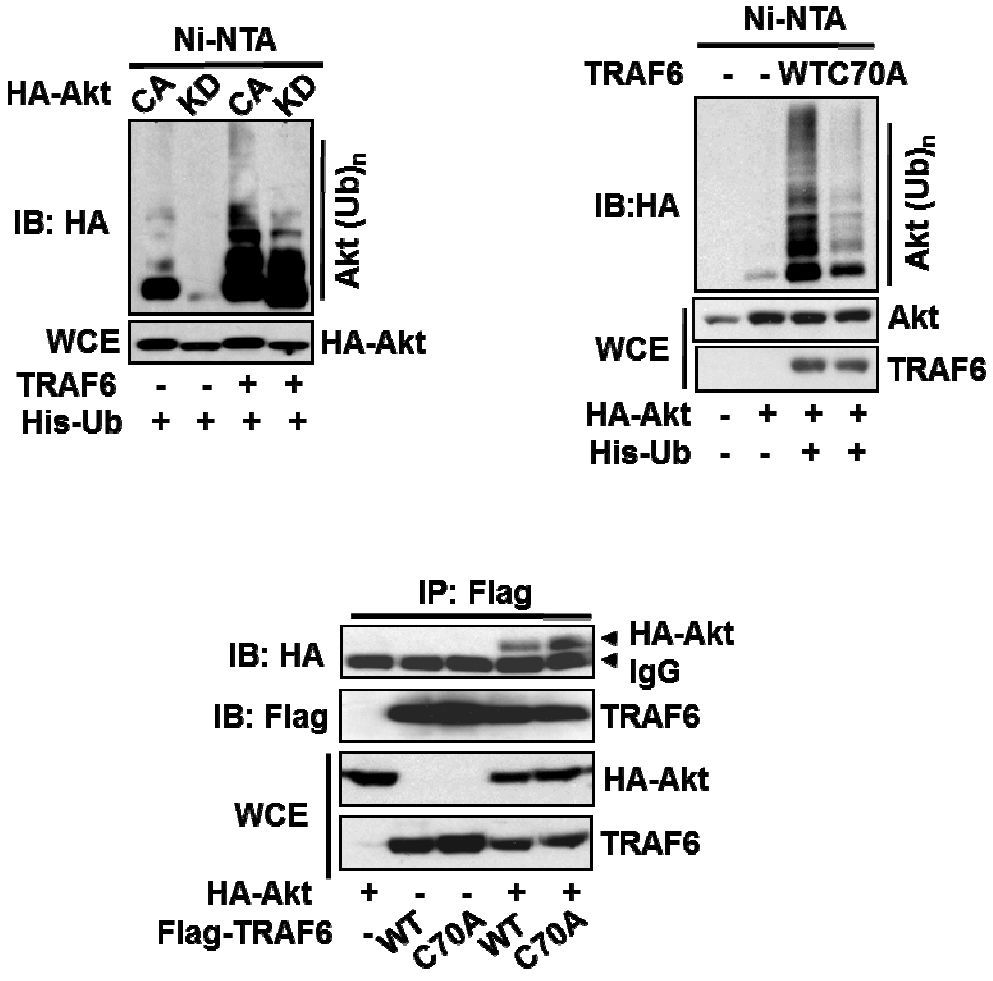

Figure 3-1. TRAF6 is an E3 ubiquitin ligase for Akt.

(E) 293T cells transfected with His-Ub and TRAF6, along with the constitutively active Akt (HA-Akt CA) or kinase dead Akt (HA-Akt KD), were lysed for in vivo ubiquitination.

(F) IB of lysed 293T cells transfected with HA-Akt and His-Ub, along with TRAF6 or TRAF6 C70A.

(G) 293T cells were transfected with HA-Akt and Flag-TRAF6 or Flag-TRAF6 C70A as indicated for 48 h and lysed for immunoprecipitation (IP) with Flag antibody, followed by immunoblot analysis.

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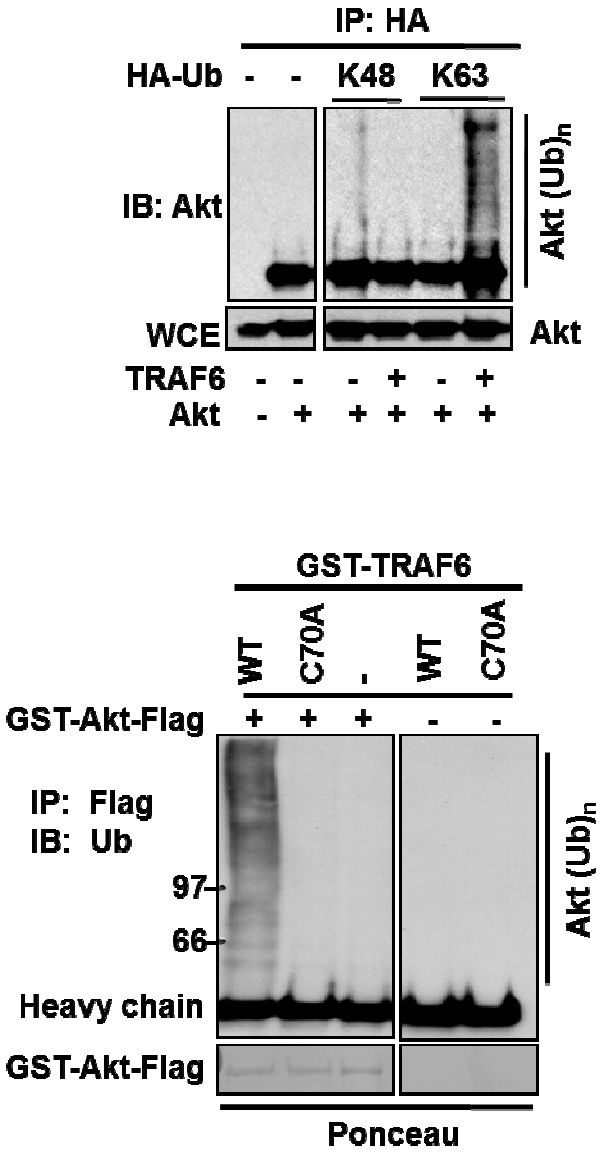

Figure 3-1. TRAF6 is an E3 ubiquitin ligase for Akt.

(H) IB of lysed 293T cells transfected with Akt and TRAF6, along with HA–Ub K48 (K48-only ubiquitin) or HA–Ub K63 (K63-only ubiquitin). IP, immunoprecipitation.

(I) GST-Akt-Flag proteins were incubated with adenosine triphosphate, E1, and E2 along with GST, GST-TRAF6, or GST–TRAF6 C70A proteins for in vitro ubiquitination of Akt.

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3-2. TRAF6 directly associates with Akt and its isoforms.

Our previous co-immunoprecipitation experiments showed that Akt associates with overexpressed TRAF6 and with TRAF6 C70A mutant (Fig. 3-1G). In addition, we detected the association between endogenous Akt and TRAF6 in cells stimulated with IGF-1 growth factor or IL-1β cytokine (Fig. 3-2A), both of which activate Akt signaling pathway. To further confirm previous results, we conducted *in vitro* protein binding assay. We found that glutathione S-transferase (GST)–tagged recombinant TRAF6 interacted with recombinant Akt directly *in vitro* (Fig. 3-2B). Since Akt has three isoforms, Akt1, Akt2 and Akt3, we next examined whether TRAF6 can promote ubiquitination on these Akt isoforms. Our result showed that ubiquitination of the Akt1 and Akt2 isoforms, but not that of Akt3, was induced by TRAF6 (Fig. 3-2C). However, co-immunoprecipitation assay showed that TRAF6 associated with all Akt isoforms (Fig. 3-2D).

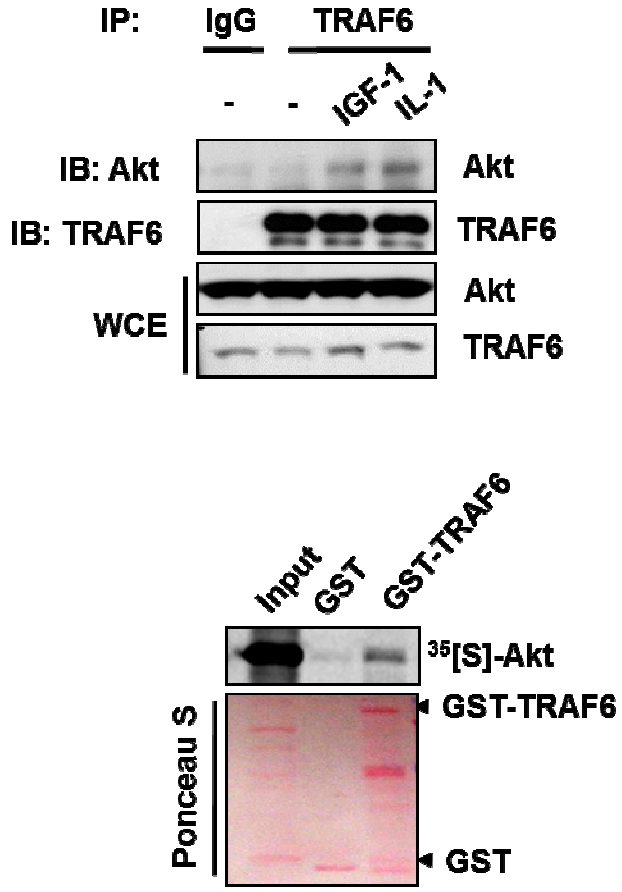

Figure 3-2. TRAF6 directly interacts with Akt *in vivo* and *in vitro*.

(A) MEFs cells were serum-starved for 1 day and treated with 100 ng/ml IGF-1 or 20 ng/ml IL-1β for 30 min, and total cell lysates were immunoprecipitated with Akt antibody, followed by Immunoblot analysis.

(B) GST-TRAF6 C70A was incubated with *in vitro* translated [S^{35}]-Akt, washed, and subjected to SDS-PAGE, followed by autoradiography.

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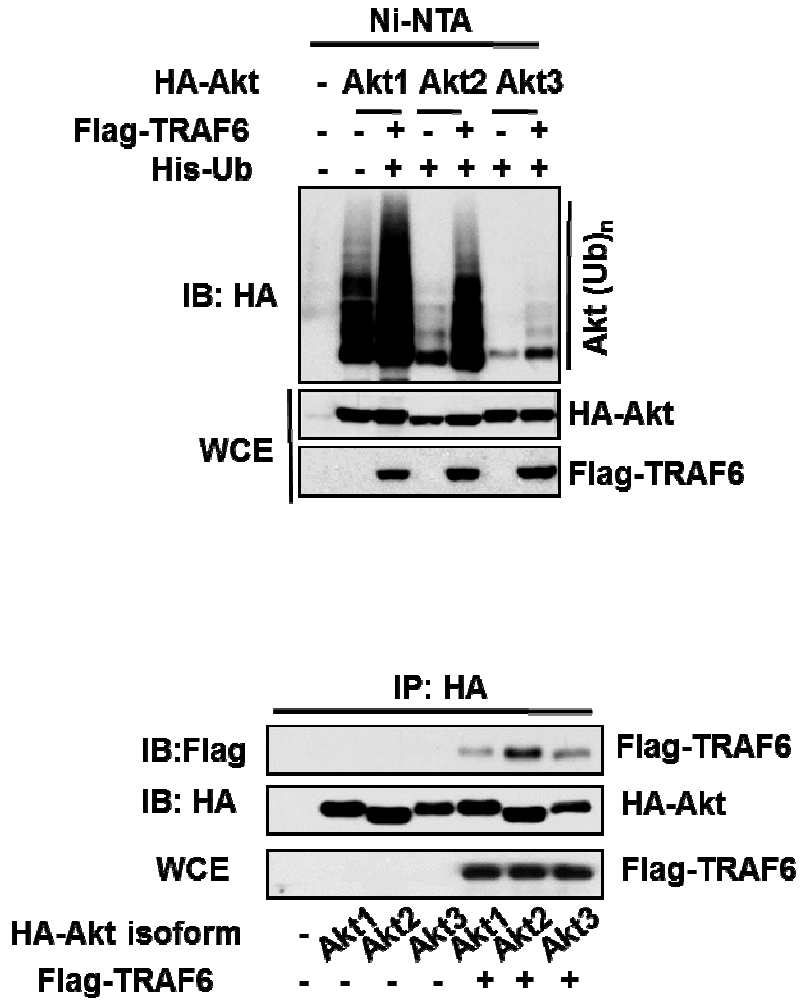

Figure 3-2. TRAF6 directly interacts with Akt *in vivo* and *in vitro*..

(C, D) 293T cells transfected with indicated plasmids and harvested for *in vivo* ubiquitination (C) and co-immunoprecipitation (D) assays.

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3-3. TRAF6 promotes phosphorylation of Akt through ubiquitination of Akt.

We next examined the function of Akt ubiquitination induced by TRAF6. Our data revealed that overexpression of TRAF6, but not that of TRAF6 C70A mutant, enhanced phosphorylation of Akt at Thr^{308} but not at Ser^{473}. This enhancement was correlated with increased Akt kinase activity toward its substrate glycogen synthase kinase 3β (GSK3β) (Fig. 3-3A) (10, 11), whereas a control HectH9 E3 ligase failed to promote phosphorylation of Akt (Fig. 3-3B). In addition, overexpression of c-IAP1 and c-IAP2, which slightly inhibited ubiquitination of Akt, reduced phosphorylation of Akt on Thr^{308} (Fig. 3-3C). These results suggest that TRAF6 may increase phosphorylation of Akt by promoting ubiquitination of Akt.

Next, we tested whether TRAF6 is required for ubiquitination and phosphorylation of Akt. To this end, we compared ubiquitination and phosphorylation of Akt in *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ primary mouse embryonic fibroblasts (MEFs) treated with inducers for Akt activation. Upon stimulation with IGF-1, endogenous ubiquitination of Akt was reduced in *Traf6*$^{-/-}$ MEFs compared with that in *Traf6*$^{+/+}$ MEFs (Fig. 3-3D). Similarly, ubiquitination of Akt was enhanced by 10% fetal bovine serum (FBS) or IL-1β in *Traf6*$^{+/+}$ MEFs but not in *Traf6*$^{-/-}$ MEFs (Fig. 3-3E). Accordingly, phosphorylation of Akt at Thr^{308} and Ser^{473} after IGF-1 treatment was decreased in *Traf6*$^{-/-}$ MEFs compared with that in *Traf6*$^{+/+}$ MEFs (Fig. 3-3F). This defect in phosphorylation of Akt in *Traf6*$^{-/-}$ MEFs was accompanied with an impairment in phosphorylation of two Akt substrates, Foxo1 and Foxo3a (11, 143). Therefore, these results suggest that TRAF6 plays a critical role in the regulation of IGF-1-mediated ubiquitination and phosphorylation of Akt.

Since our previous results showed that TRAF6 is essential for IGF-1-induced ubiquitination and phosphorylation of Akt, we next determined whether TRAF6 is directly involved in IGF-1-mediated Akt activation. We found that ubiquitination of TRAF6, which represents TRAF6 E3 ligase activity, was also induced by IGF-1 stimulation in two different cell lines (Fig. 3-3G). In addition, TRAF6 interacted with IGF-1 receptor (IGF-1R) in serum-deprived conditions but disruption of this interaction was found after IGF-1 treatment (Fig. 3-3H). Therefore, our findings suggest that activated IGF-1 receptor by IGF-1 stimulation may directly engage TRAF6 activation and further promote ubiquitination and phosphorylation of Akt.

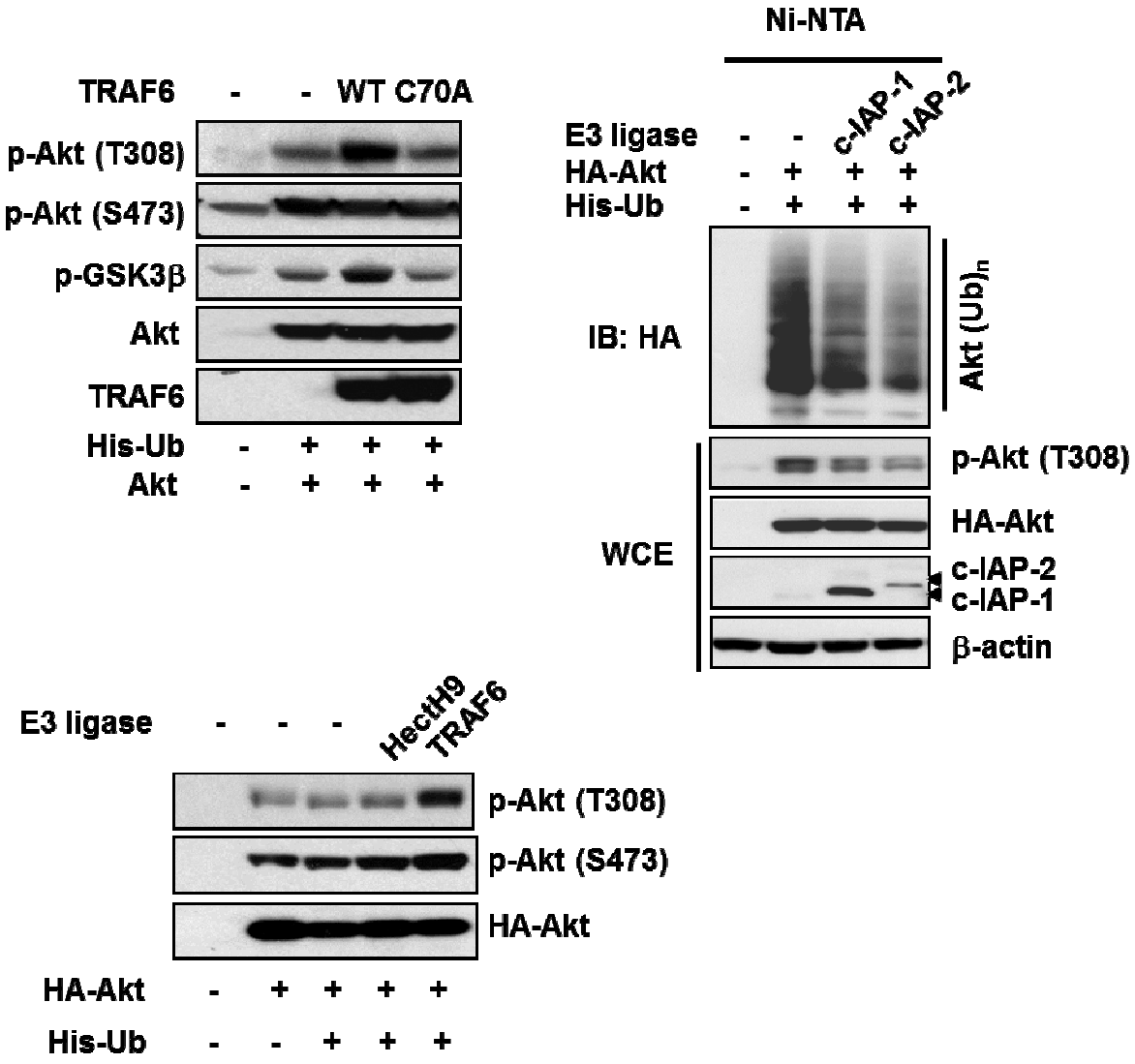

Figure 3-3. TRAF6 is required for ubiquitination and phosphorylation of Akt.

(A) WCE from293T cells transfected with indicated plasmids was collected for IB analysis.

(B) WCE from 293T cells transfected with vector, TRAF6, or HectH9 for 48 h were collected for immunoblot analysis.

(C) 293T cells transfected with various plasmids were lysed for *in vivo* ubiquitination and IB analysis.

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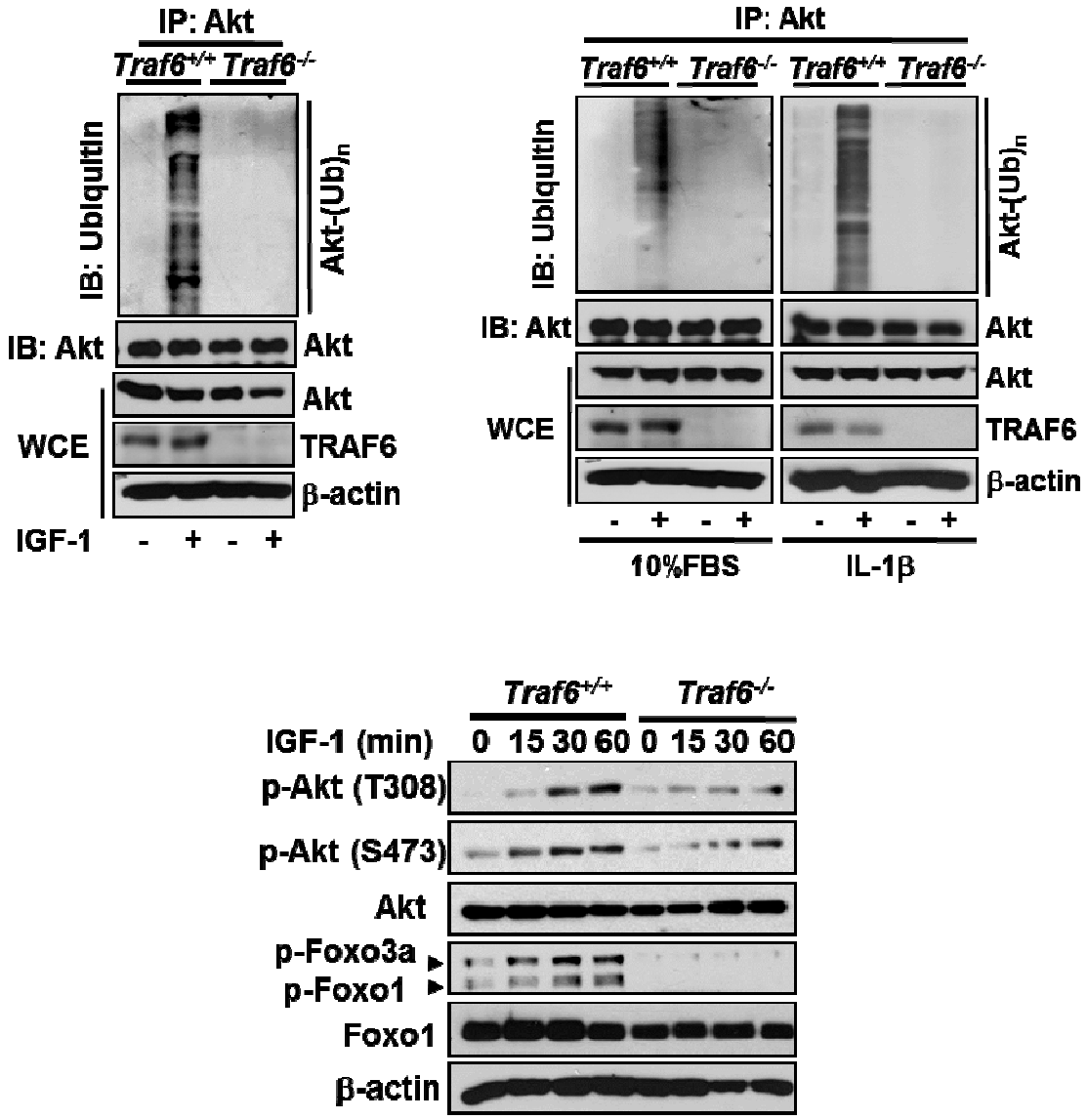

Figure 3-3. TRAF6 is required for ubiquitination and phosphorylation of Akt.

(D, E) *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ MEFs were serum-starved for 1 day and treated with/without IGF-1 (A) or 10% FBS or 20 ng/ml IL-1β (B) for 30 min; WCE were collected for immunoprecipitation with Akt, followed by IB analysis.

(F) MEFs were serum-starved, treated with IGF-1 for various time points, and harvested for IB analysis.

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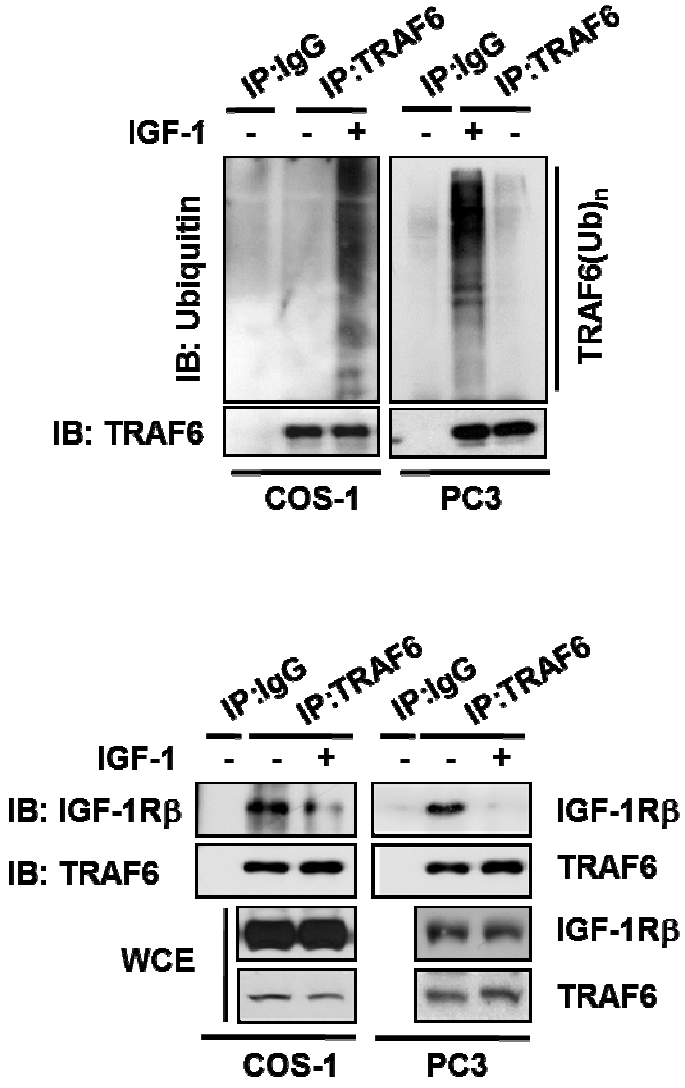

Figure 3-3. TRAF6 is required for ubiquitination and phosphorylation of Akt.

(G, H) COS-1 and PC-3 cells were serum-starved for 1 day and treated with or without 100 ng/ml of IGF-1; WCE were collected for immunoprecipitation with TRAF6, followed by immunoblot analysis with anti-ubiquitin antibody (G) or anti-IGF-1Rβ antibody (H).

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3-4. TLR4/IL-1R signaling cooperates with growth factor receptor signaling to promote Akt activation

Phosphorylations of Akt at Thr^{308} and Ser^{473} were also decreased in *Traf6*$^{-/-}$ MEFs treated with 10% FBS (Fig. 3-4A). In contrast, phosphorylations of extracellular signal-regulated protein kinase (ERK1) and ERK2 were similar in wild-type and *Traf6*$^{-/-}$ MEFs (Fig. 3-4A). These results suggest that TRAF6 selectively regulates activation of Akt.

The endotoxin LPS and cytokine IL-1 can activate the TLR4/IL-1R signal transduction pathway (99). Because TRAF6 is a central modulator for TLR4/IL-1R signal transduction pathway (99, 140), we examined whether LPS- or IL-1β-mediated phosphorylation of Akt acts through TRAF6. LPS or IL-1β treatment promoted phosphorylation of Akt at Thr^{308} in *Traf6*$^{+/+}$ MEFs in the presence of 10% FBS. However, this effect was inhibited in *Traf6*$^{-/-}$ MEFs (Fig. 3-4B and 3-4C). In serum-starved condition (0.1% FBS), neither LPS nor IL-1β was sufficient to initiate phosphorylation of Akt at either Thr^{308} or Ser^{473} in primary MEFs and IMR90 cell line (Fig. 3-4D). Thus, these results suggest that LPS and IL-1β may require cooperation with growth-factor receptor signaling to induce activation of Akt.

Reconstitution of TRAF6 expression in *Traf6*$^{-/-}$ MEFs rescued the deficiency of phosphorylation of Akt in cells treated with IGF-1 or IL-1β, whereas reconstitution of TRAF6 C70A mutant did not (Fig. 3-4E and Fig. 3-4F). Therefore, these results suggest that TRAF6 is required for phosphorylation and activation of Akt through induction of Akt ubiquitination.

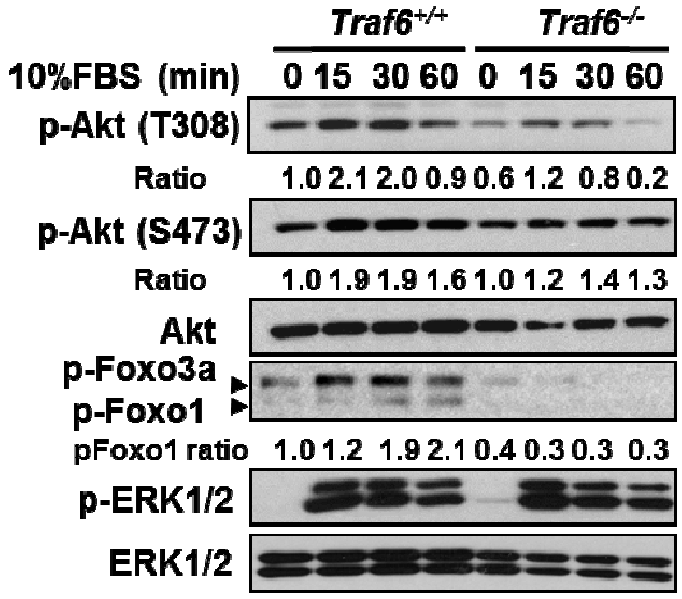

Figure 3-4. TLR4/IL-1R signaling collaborates with growth factor receptor signaling in regulating activation of Akt.

(A) *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ MEFs were serum-starved for 1 day, treated with 10% FBS for various time points, and harvested for immunoblot analysis.

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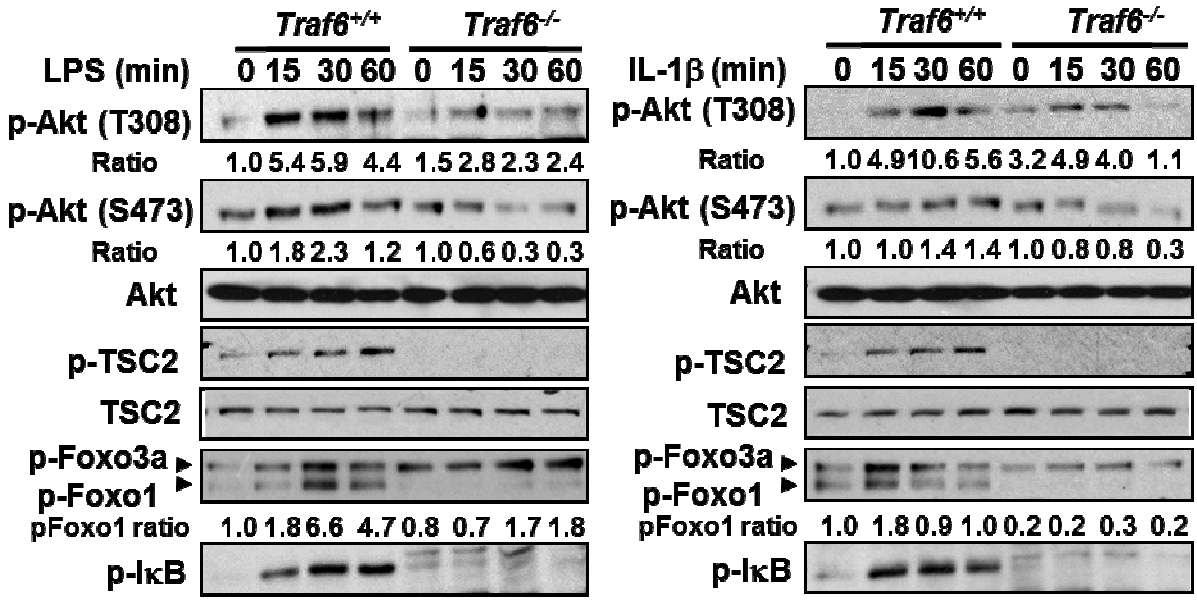

Figure 3-4. TLR4/IL-1R signaling collaborates with growth factor receptor signaling in regulating activation of Akt.

(B, C) *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ MEFs cultured in 10% FBS were treated with 10 μg/ml LPS (B) or 20 ng/ml IL-1β (C) for various times as indicated and harvested for immunoblot analysis.

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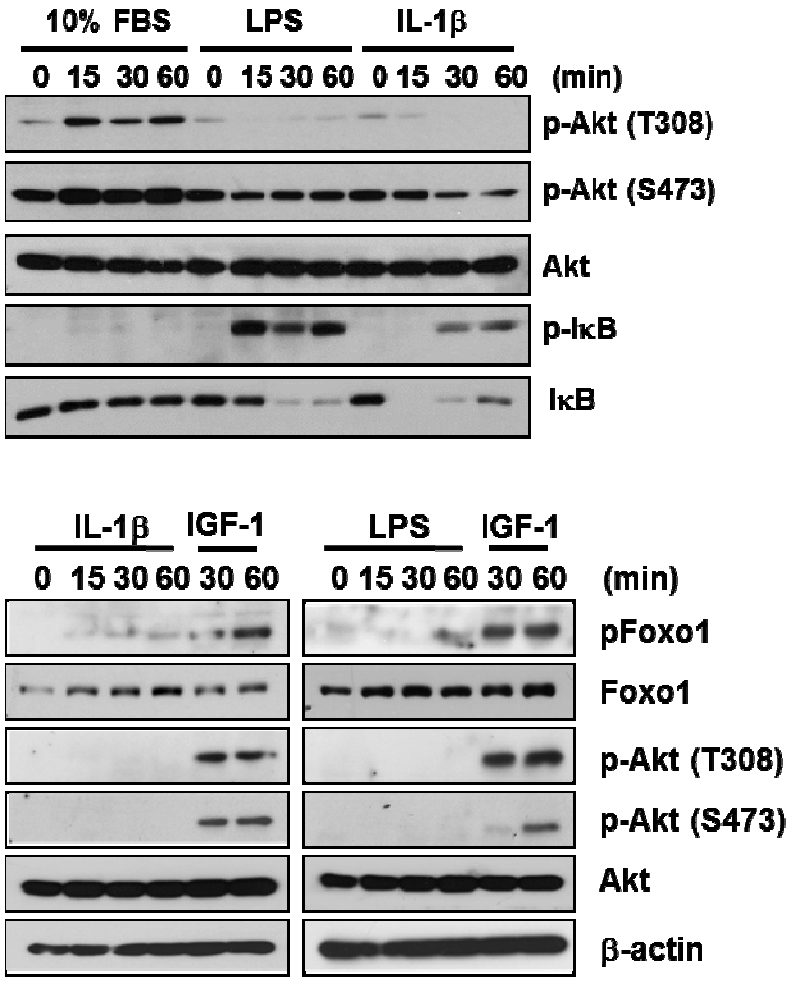

Figure 3-4. TLR4/IL-1R signaling collaborates with growth factor receptor signaling in regulating activation of Akt.

(D) Primary MEFs or IMR90 cell lines were serum-starved for 1 day, treated with 10% FBS, 100 ng/ml IGF-1, 10 μg/ml LPS, or 20 ng/ml IL-1β in the absence of 10% FBS for various times as indicated and harvested for IB analysis.

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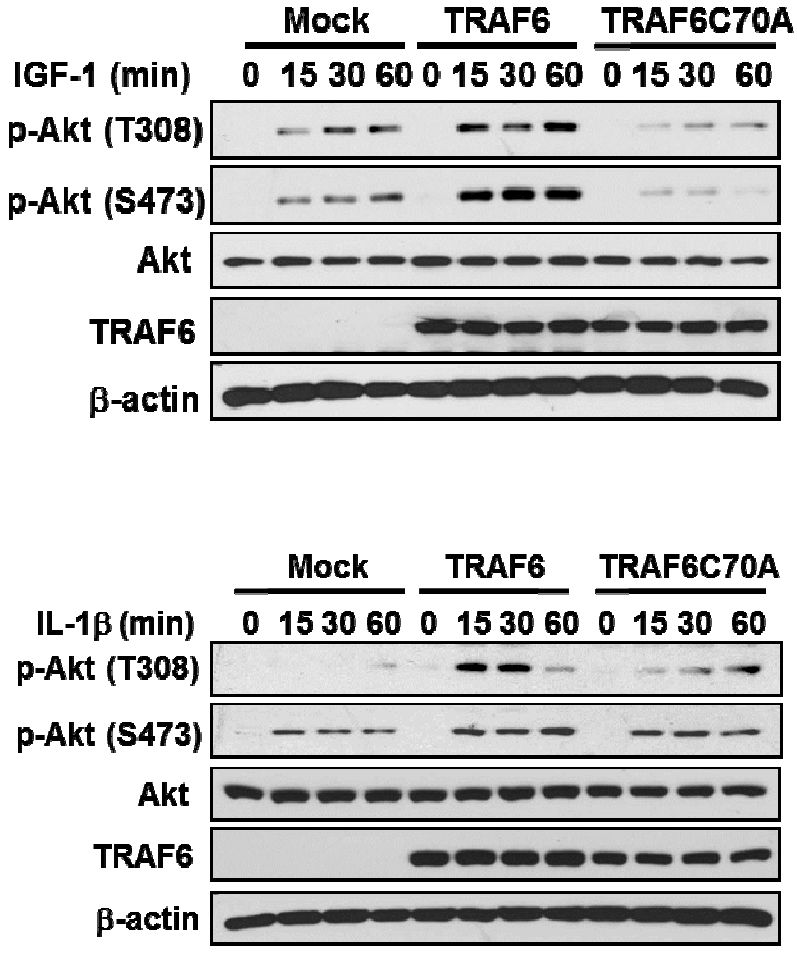

Figure 3-4. TLR4/IL-1R signaling collaborates with growth factor receptor signaling in regulating activation of Akt.

(E, F) Primary *Traf6*$^{-/-}$ MEFs infected with mock, TRAF6, or TRAF6 C70A mutant were treated with 100 ng/ml IGF-1 (E) or 20 ng/ml IL-1β (F) at various time points and harvested for IB analysis.

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3-5. TRAF6 regulates cell survival through activating Akt

Since Akt signaling activation controls cell survival and apoptosis (11, 143), we determined whether *Traf6* deficiency sensitized cells to apoptosis after serum withdrawal. Our result demonstrated that apoptosis in *Traf6*$^{-/-}$ MEFs was higher than in *Traf6*$^{+/+}$ MEFs in the presence and absence of serum (Fig. 3-5A). Furthermore, the active, cleaved form of caspase-3 which is a critical regulator of apoptosis, was more abundant in *Traf6*$^{-/-}$ MEFs than in *Traf6*$^{+/+}$ MEFs (Fig. 3-5B). In addition, reconstitution of TRAF6, but not TRAF6 C70A mutant, prevented *Traf6*$^{-/-}$ MEFs from apoptosis (Fig. 3-5C). However, a constitutively active form of Akt (myristoylated Akt) partially prevented cells from apoptosis (Fig. 3-5C), suggesting that other signaling pathways may be also engaged in the cell survival response.

Since apoptosis inducers, including DNA damage agents, also promote phosphorylation and activation of Akt (144, 145), we examined whether TRAF6 was also involved in this process. Doxorubicin (Dox)- or cisplatin (Cis)-mediated phosphorylation of Akt at Thr^{308} in wild-type MEFs was reduced in *Traf6*$^{-/-}$ MEFs (Fig. 3-5D). The deficiency of phosphorylation of Akt was accompanied with increased activation of caspase-3 in *Traf6*$^{-/-}$ MEFs (Fig. 3-5E). Accordingly, these results suggest that TRAF6 promotes activation of Akt in response to serum starvation- or DNA damage agents-induced apoptosis.

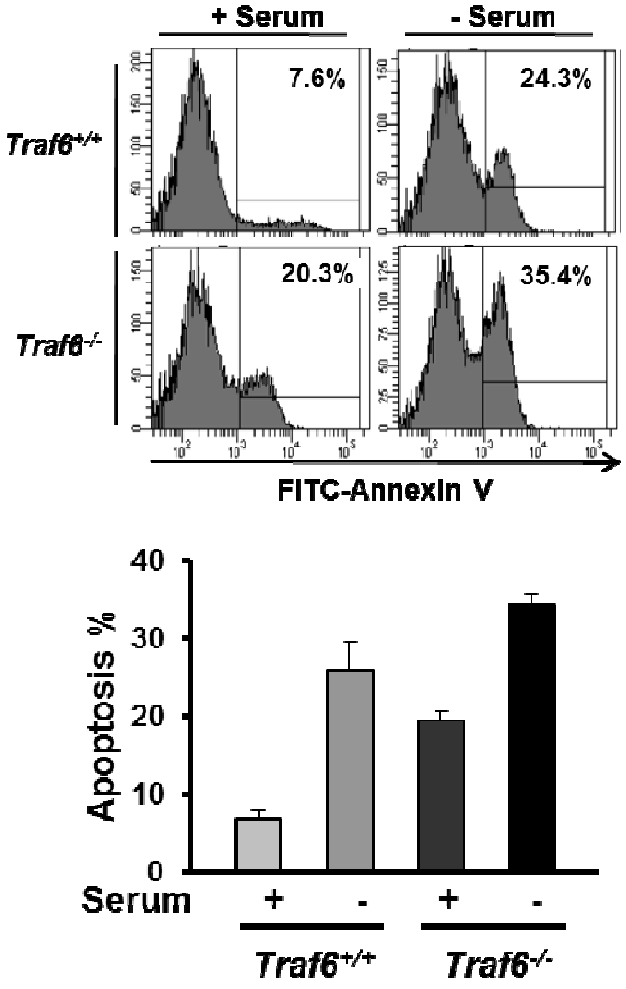

Figure 3-5. TRAF6 is required for Akt-mediated cell survival in response to serum-starvation and DNA damage.

(A) MEFs were cultured in 10% FBS or serum-starved for 2 days, and apoptosis was determined by annexin V staining, followed by flow cytometry analysis. Results are presented as mean values ± SD.

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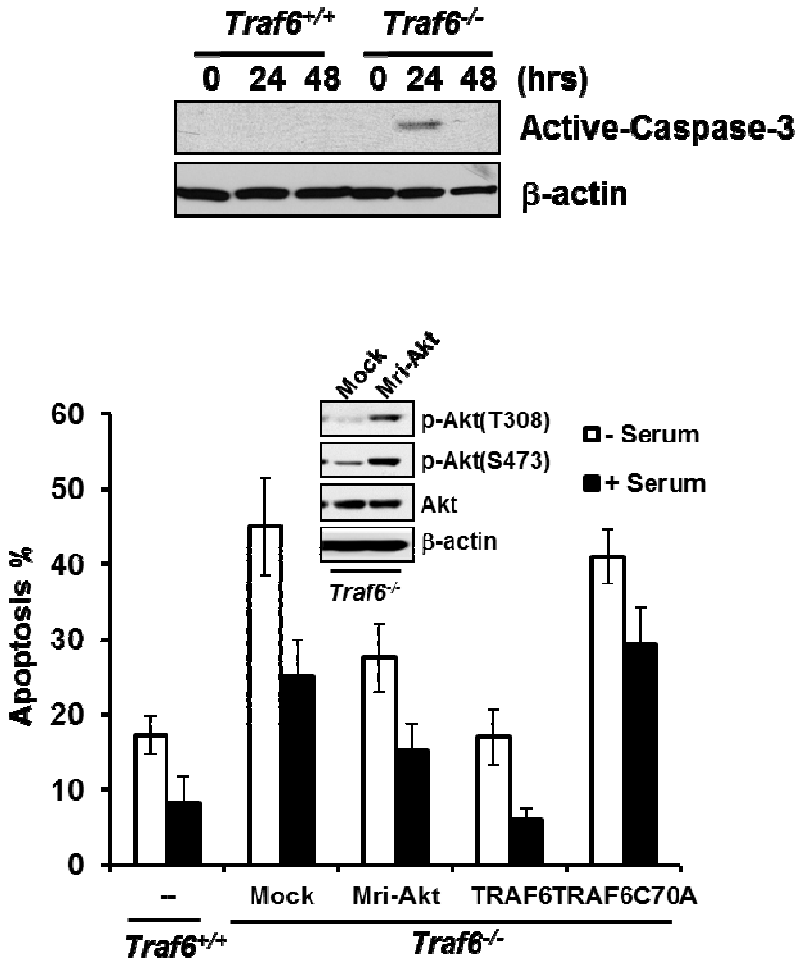

Figure 3-5. TRAF6 is required for Akt-mediated cell survival in response to serum-starvation and DNA damage.

(B) MEFs were serum-staved for various times and harvested for IB analysis.

(C) MEFs were infected with mock, constitutively active Akt (Mri-Akt), TRAF6, or TRAF6 C70A; and apoptosis and IB analysis were determined. Results are presented as mean values ± SD.

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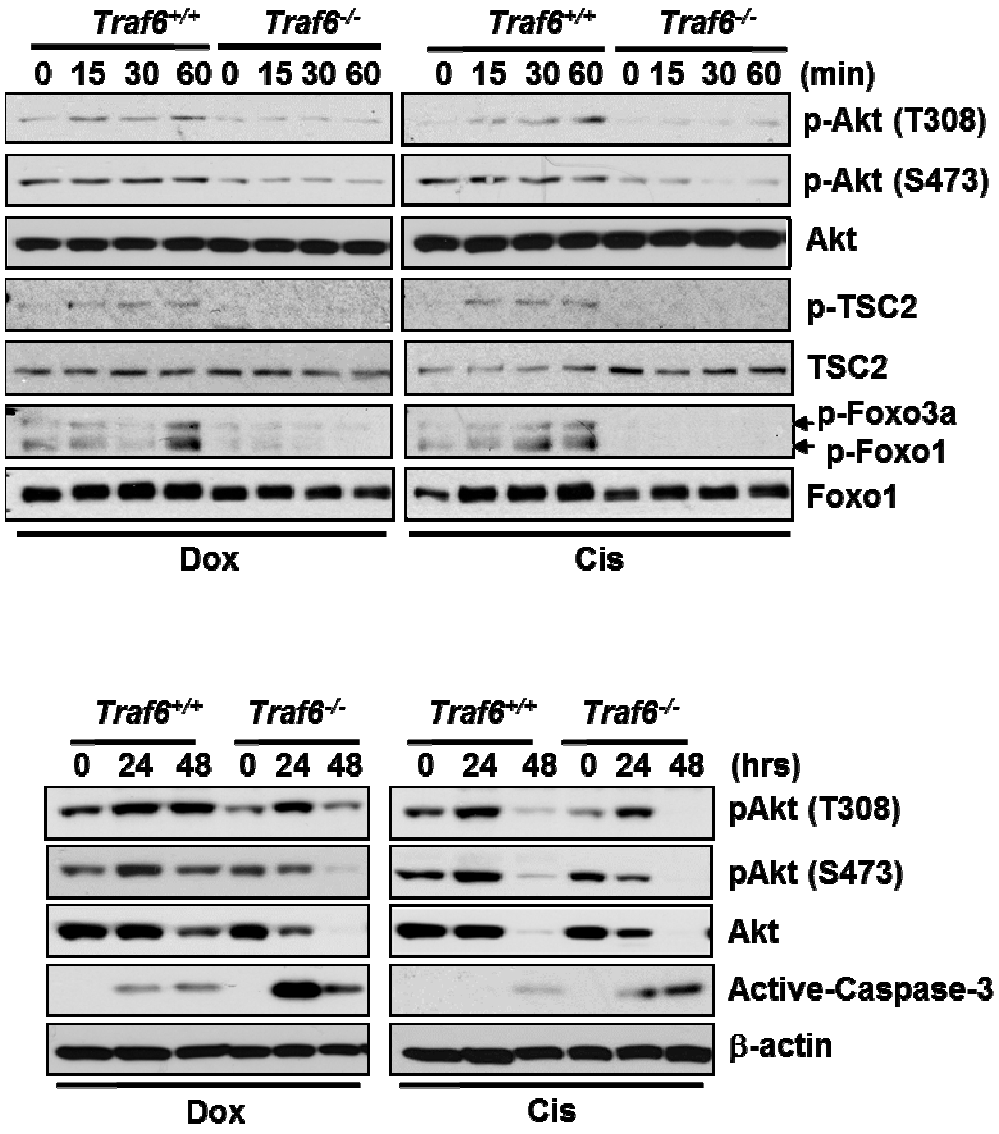

Figure 3-5. TRAF6 is required for Akt-mediated cell survival in response to serum-starvation and DNA damage.

(D, E) *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ MEFs were treated with Doxorubicin (Dox) and Cisplatin (Cis) for various times and harvested for immunoblot analysis.

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3-6. TRAF6 regulates steady-state Akt activity *in vivo*.

Next, we used TRAF6 deficiency mouse model to further corroborate our previous findings of the role of TRAF6 in regulating Akt activation in *in vivo*. TRAF6 is expressed in most mouse tissues, such as skeletal muscle, heart and kidney (146, 147). We compared Akt activity in skeletal muscle and heart tissues obtained from wild-type and *Traf6*$^{-/-}$ mice, respectively. The result showed that steady-state Akt activity level in heart muscle, but not skeletal muscle, was lower in *Traf6*$^{-/-}$ mice than in wild-type mice (Fig. 3-6A). In addition, activation of Akt in animals injected with IGF-1 was reduced in both forms of muscles in *Traf6*$^{-/-}$ mice (Fig. 3-6B). Therefore, these results suggest that TRAF6 plays a critical role in Akt activation *in vivo*.

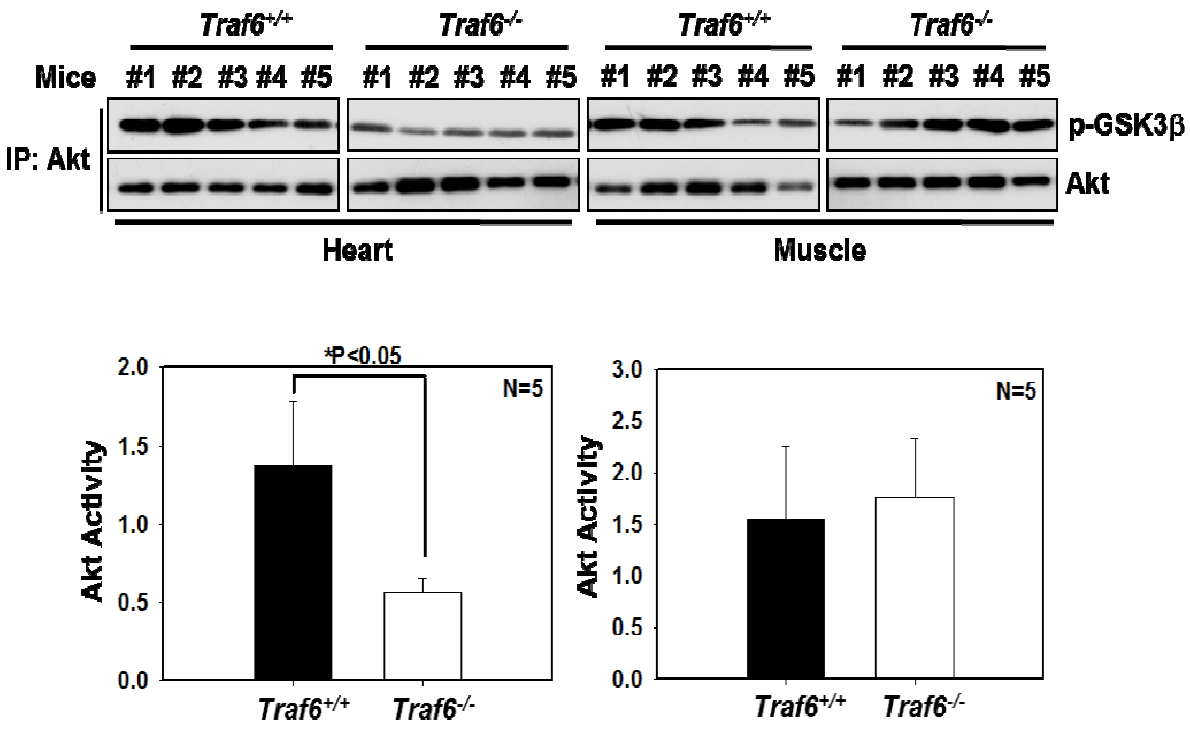

Figure 3-6. TRAF6 is required for both steady-state and IGF-1-induced Akt activation *in vivo*.

(A) Heart and skeletal muscle isolated from *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ mice (n=5) was lysed and subjected to an in vitro Akt kinase assay and immunoblot analysis. The graph (lower panel) represents the averaged Akt activity from total 5 mice. Results are presented as mean values ± S.D. *p<0.05, using Student's t-test.

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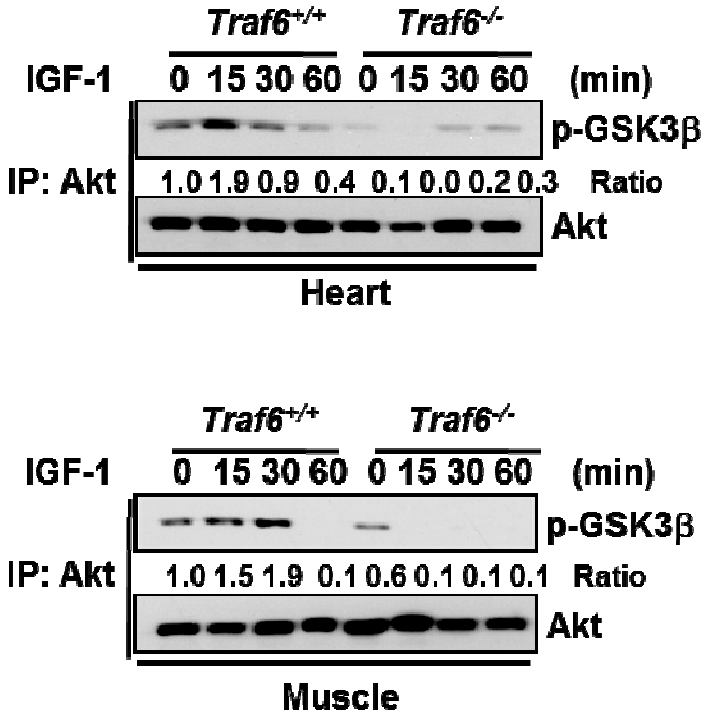

Figure 3-6. TRAF6 is required for both steady-state and IGF-1-induced Akt activation *in vivo*.

(B) Heart and skeletal muscles isolated from *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ mice (n = 4) injected with IGF-1 at various time points were subjected to an *in vitro* Akt kinase assay and IB analysis.

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3-7. TRAF6 ubiquitinates Akt at the PH domain of Akt.

Because the pleckstrin homology (PH) domain of Akt is critical for PIP3 phospholipid binding and protein-protein interaction (10, 148), we analyzed whether the PH domain of Akt influenced TRAF6-mediated ubiquitination of Akt. Indeed, TRAF6 failed to promote ubiquitination of the Akt mutant devoid of the PH domain (Fig. 3-7A). Of the six lysine residues (K8, K14, K20, K30, K39, K64) within the PH domain of Akt, mutation on either K8 or K14 to arginine (R) most substantially reduced Akt ubiquitination and Akt phosphorylation at Thr^{308} and Ser^{473} (Fig. 3-7B). In addition, amino acid sequence alignment and analysis for the PH domain of Akt revealed that the K8 and K14 residues are well conserved from Drosophila to humans (Fig. 3-7C), suggesting that the ubiquitination site of Akt may be evolutionarily conserved.

Next we tested whether ubiquitination within the PH domain of Akt affects Akt interaction with PIP3. The result of PIP3 binding assay showed that Akt K8R mutant bound effectively to isolate PIP3, but Akt K14R mutant did not (Fig. 3-7D). The K14 residue lies within the PIP3 phospholipid-binding pocket (21, 56, 149). In addition, overexpression of TRAF6 did not enhance the binding of Akt to PIP3 (Fig. 3-7D). Thus, Akt ubiquitination by TRAF6 appears not to influence PIP3 lipid binding, and the defect in phosphorylation of Akt K8R mutant is not due to its impairment in PIP3 binding.

A mutation in the PH domain [$Glu^{17} \rightarrow Lys^{17}$, E17K] of Akt has been identified in human cancer patients, including those with breast and colon cancers (56). This cancer-associated Akt mutant exhibited constitutive Akt phosphorylation at Thr^{308} but not at Ser^{473} and had greater oncogenic potential. Since this mutant gains an additional lysine within the PH domain of Akt, we inferred that this mutant may have higher ubiquitination level, leading to hyper-activation of this mutant. To test this hypothesis, we next examined ubiquitination of Akt E17K mutant compared with Akt wild-type. The result showed that basal ubiquitination of the E17K mutant was much higher than that of wild-type Akt (Fig. 3-7E). In addition, overexpression of TRAF6 still increased ubiquitination of this mutant but to a lesser extent than wild-type Akt (Fig. 3-7E). The E17K mutant displayed higher Akt phosphorylation at Thr^{308} but not at Ser^{473} (56), however this was not increased by TRAF6 overexpression (Fig. 3-7E). To further confirm the role of ubiquitination in Akt E17K mutant, we generated and tested the Akt K8R/E17K mutant. The result supported our previous findings that ubiquitination of a K8R/E17K Akt mutant *in vivo* was reduced and correlated with a reduction in phosphorylation of Akt Thr^{308} (Fig. 3-7F). Thus, increased Akt ubiquitination apparently contributes to the hyper-activation of Akt observed in the Akt E17K mutant.

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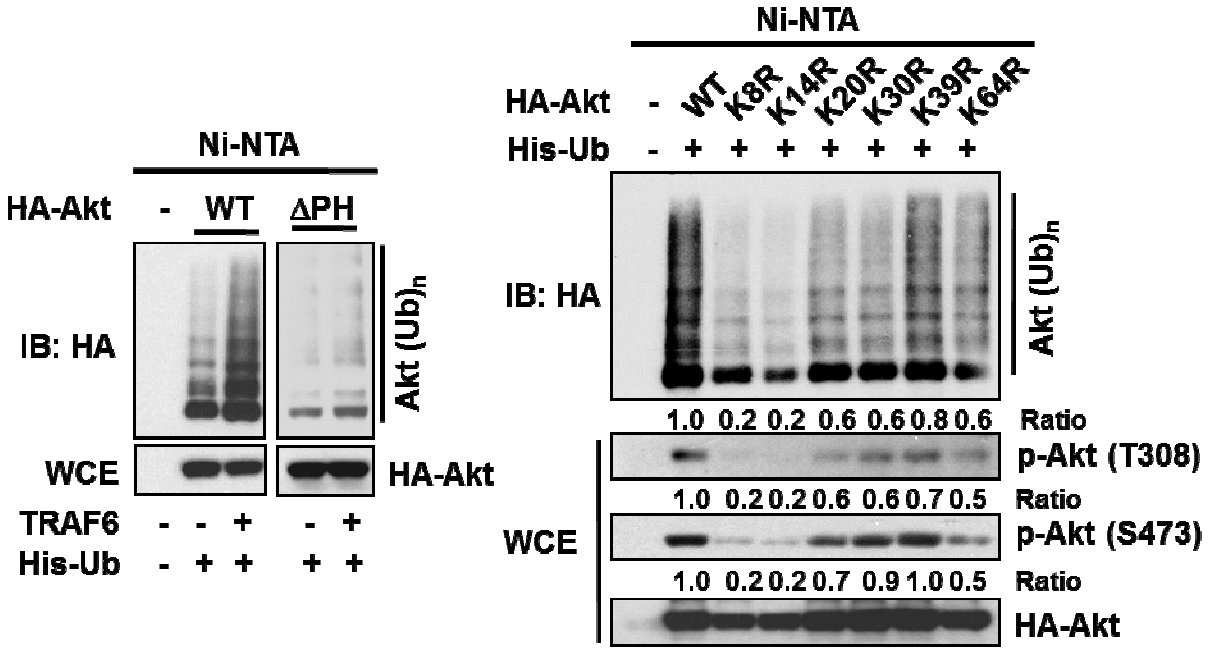

Figure 3-7. The PH domain of Akt is required for Akt ubiquitination by TRAF6.

(A) IB of lysed 293T cells transfected with His-Ub along with HA-Akt or HA–Akt ΔPH.

(B) IB of 293T cells transfected with His-Ub along with HA-Akt or various HA-Akt mutants and lysed for ubiquitination and phosphorylation of Akt.

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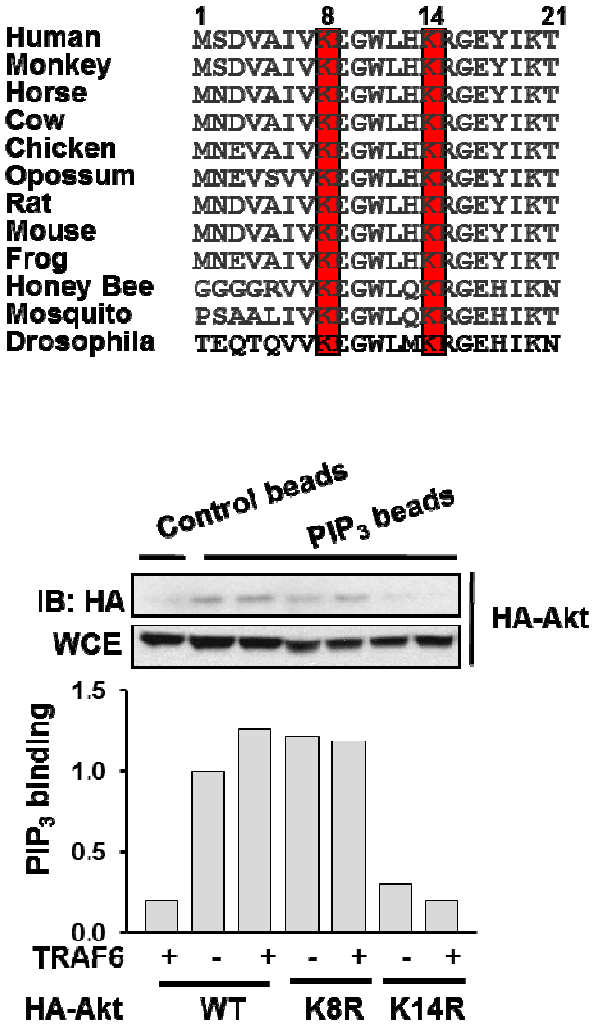

Figure 3-7. The PH domain of Akt is required for Akt ubiquitination by TRAF6.

(C) Conservation of the K8 and K14 residues within the PH domain of Akt among the species.

(D) WCE from 293T cells transfected with HA-Akt or various Akt mutants were incubated with control or PIP3 beads for overnight, washed, and subjected to IB analysis. The PIP3 binding was calculated as the ratio between amounts of Akt bound with PIP3 beads and total amounts of Akt.

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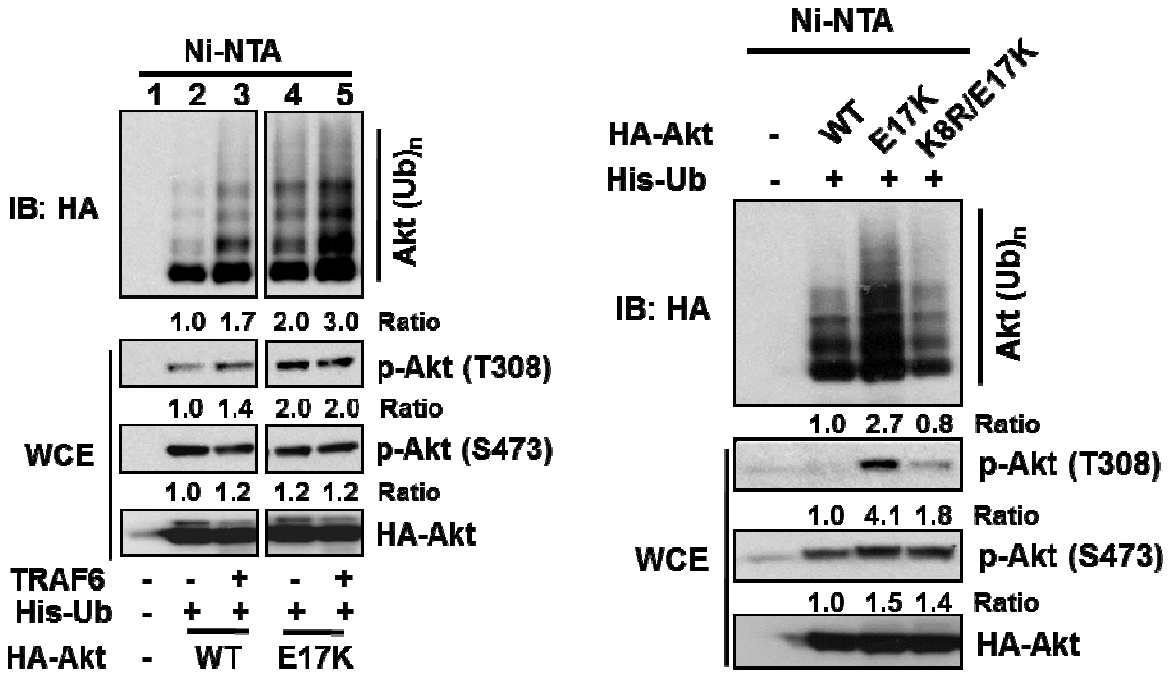

Figure 3-7. The PH domain of Akt is required for Akt ubiquitination by TRAF6.

(E) IBs of lysed 293T cells transfected with TRAF6, His-Ub, HA-Akt WT or E17K mutant.

(F) IBs of lysed 293T cells transfected with His-Ub, HA-Akt WT, E17K or K8R/E17K mutant.

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3-8. TRAF6-mediated ubiquitination of Akt promotes recruitment of Akt to the plasma membrane.

Because K63-linked ubiquitination regulates protein trafficking among different cellular compartments, we tested whether TRAF6 influenced membrane recruitment of Akt. Indeed, overexpression of TRAF6 increased Akt membrane localization, which correlated with an increase in phosphorylation and ubiquitination of Akt (Fig. 3-8A). In addition, IGF-1-induced membrane localization and phosphorylation of Akt at Thr^{308} in wild-type MEFs was abolished in *Traf6*$^{-/-}$ MEFs (Fig. 3-8B). Thus, TRAF6 is required for Akt membrane recruitment and phosphorylation upon IGF-1 stimulation.

We also compared the membrane recruitment of wild-type Akt and Akt mutants (K8R, K14R, E17K and K8R/E17K), which affects Akt ubiquitination. The result showed that membrane recruitment of Akt K8R and K14R upon IGF-1 treatment was reduced (Fig. 3-8C). Furthermore, the Akt E17K mutant localized to the membrane, even without IGF-1 stimulation, although IGF-1 stimulation further increased membrane recruitment (Fig. 3-8C). In contrast, the Akt K8R/E17K mutant showed impaired association with the membrane (Fig. 3-8C). Accordingly, this result suggests that Akt ubiquitination contributes to membrane recruitment and phosphorylation of Akt.

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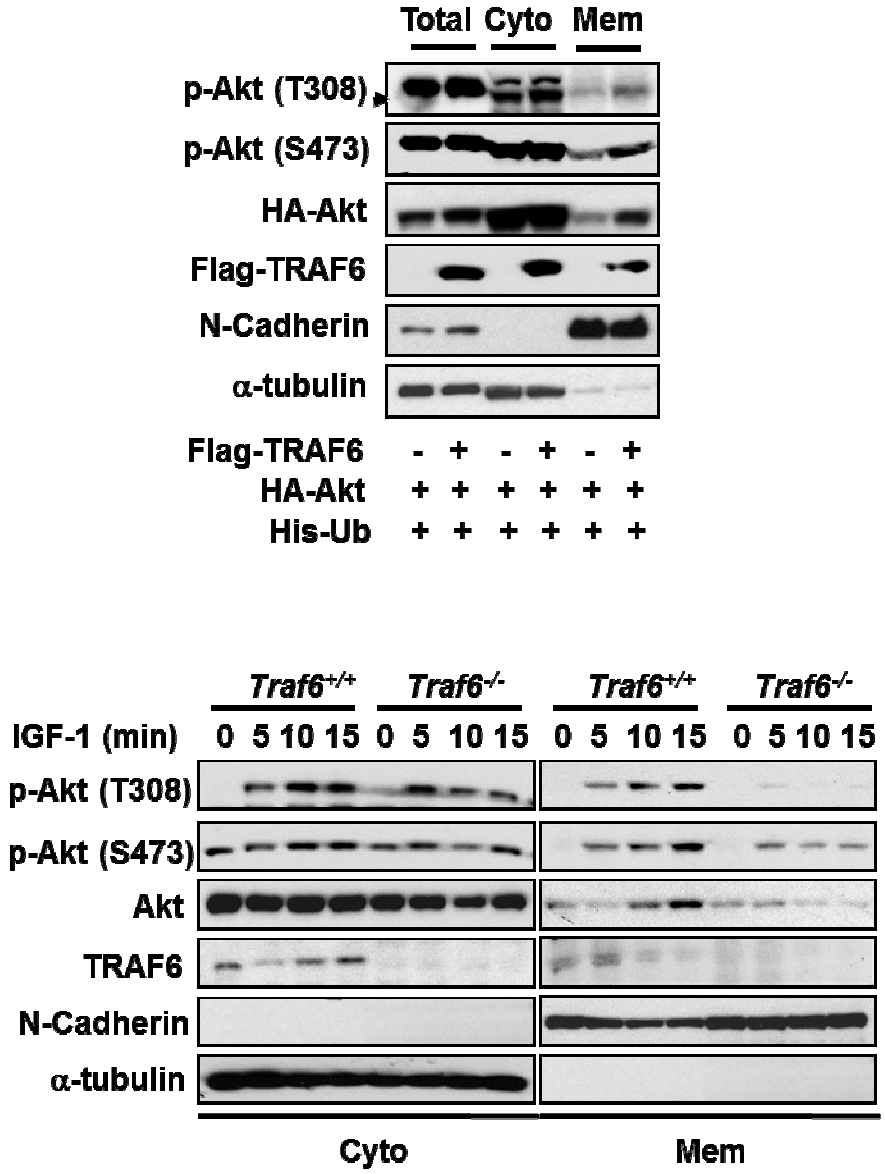

Figure 3-8. Ubiquitination of Akt is required for membrane recruitment of Akt.

(A) The membrane (Mem) and cytosolic (Cyto) fractions from 293T cells transfected with mock or TRAF6 were subjected to IB analysis.

(B) MEFs were serum-starved and treated with IGF-1, and the membrane and cytosolic fractions were isolated for IB analysis.

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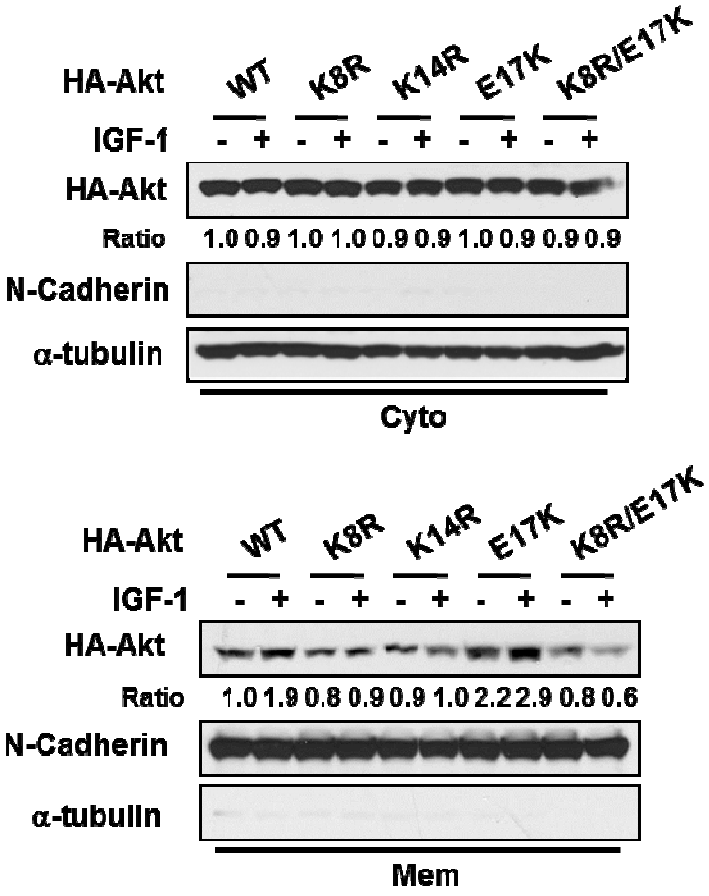

Figure 3-8. Ubiquitination of Akt is required for membrane recruitment of Akt.

(C) COS-1 cells were transfected with indicated plasmids, serum-starved, and treated with IGF-1 for 15 min; and the cytosolic (Cyto) and membrane (Mem) fractions were isolated for IB analysis.

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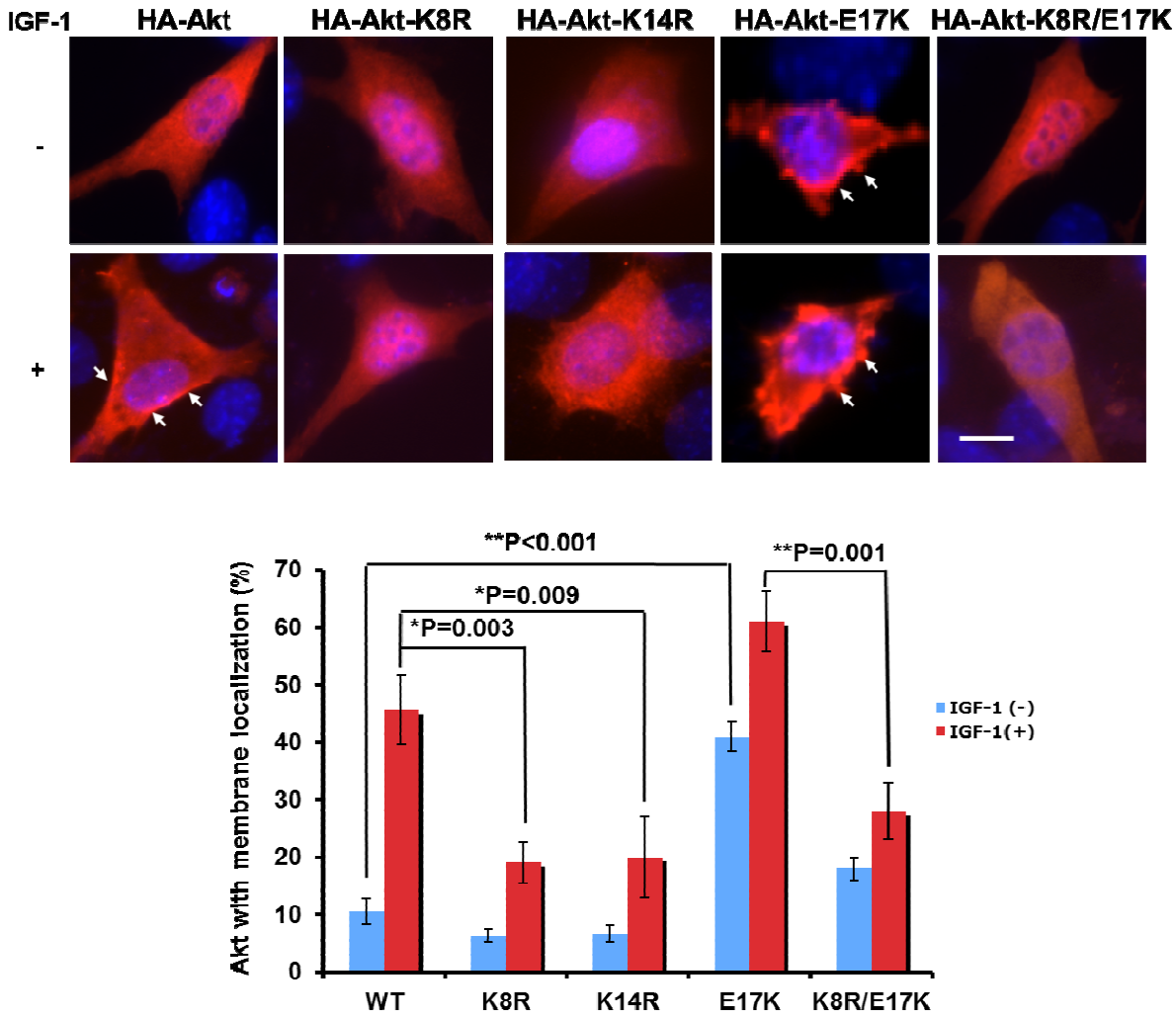

Figure 3-8. Ubiquitination of Akt is required for membrane recruitment of Akt.

(D) NIH3T3 cells transfected with HA-Akt or various Akt mutants for 24 h were serum-starved with 0.1% FBS for 1day, treated with 100 ng/ml of IGF-1 for 15 min, and fixed for immunofluorescence. The Arrow indicates the membrane localization of Akt. The scale bar represents 10 μm. Quantification of the experiments is shown in lower panel. Results are presented as mean values ± S.D. *p<0.05, **p<0.001 using Student's t-test.

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3-9. TRAF6 is required for prostate cancer development.

Since deregulated Akt activity can contribute to human cancer development (9, 12), we depleted TRAF6 protein expression in PC-3 prostate cancer cells by using short hairpin RNAs (shRNAs) to determine whether TRAF6 is also involved in tumorigenesis through regulating Akt activation. TRAF6 deficiency in PC-3 cells decreased phosphorylation of Akt at Thr^{308} and Ser^{473} (Fig. 3-9A). Furthermore, in cells treated with IGF-1, phosphorylation of Akt in TRAF6 deficient cells was impaired (Fig. 3-9B). In addition, in xenograft tumor models, the two stable TRAF6 deficient cells showed lower tumorigenic potential than control cells (Fig. 3-9C). Thus, these results suggest that TRAF6 appears to influence tumorigenesis in this model probably through regulating ubiquitination and activation of an oncogenic protein Akt.

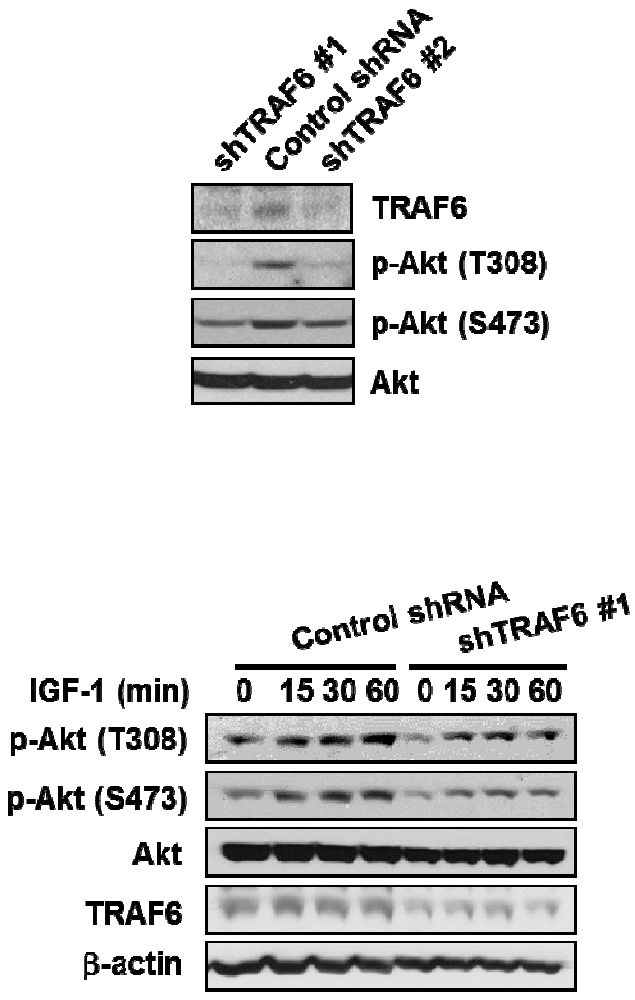

Figure 3-9. TRAF6 silencing inhibits tumorigenic potential of prostate cancer cells.

(A, B) PC-3 cells silenced with control or TRAF6 shRNAs were harvested for IB analysis or serum-starved, treated with IGF-1 for various times, and harvested for IB analysis.

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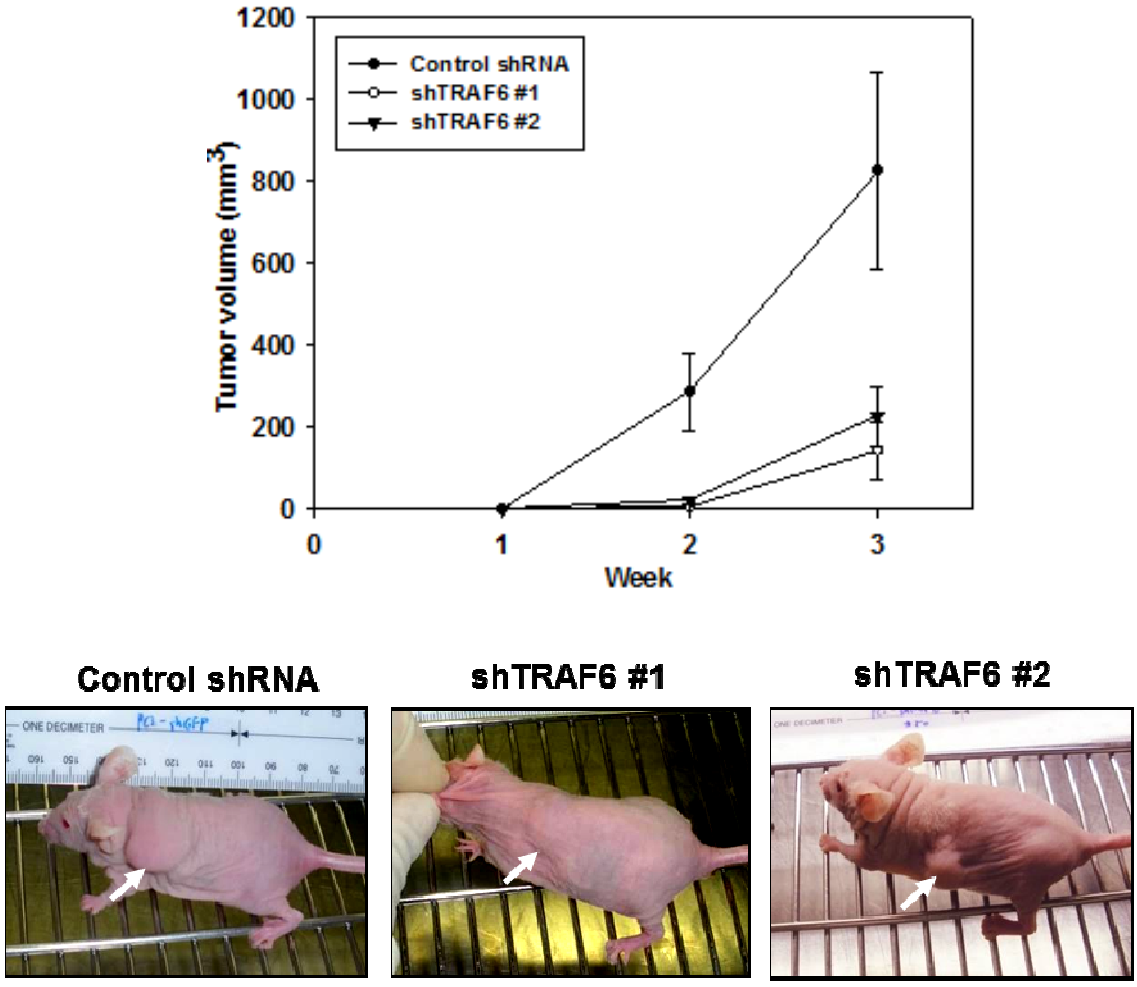

Figure 3-9. TRAF6 silencing inhibits tumorigenic potential of prostate cancer cells.

(C) PC-3 cells silenced with control or TRAF6 shRNAs were injected into nude mice (n = 6 for each group) and monitored for tumorigenesis. Results are presented as mean values ± SD. *P < 0.05, using Student's t test.

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SUMMARY

In this study, we have several important discoveries. First, we have demonstrated that Akt can be conjugated with non-proteolytic K63-linked ubiquitination, and we have identified that TRAF6 as an ubiquitin E3 ligase for Akt ubiquitination. Second, we have determined that this novel modification on Akt is important for Akt membrane recruitment, phosphorylation and activation upon stimulation with growth factor signaling. Third, we have connected K63-linked ubiquitination of Akt to human cancer development by examining a human cancer-associated Akt E17K mutant which exhibited high level of ubiquitination correlated with its hyper-activation. Fourth, we also demonstrated that TRAF6 is essential for prostate cancer development. Our results expand the known functions of TRAF6 to include the PI3K/Akt oncogenic signaling pathways. Therefore, we suggest that TRAF6 may be a previously uncharacterized oncogene that may serve as an important therapeutic target for human cancers.

Based on our previous findings, we propose a hypothetical model as shown in Figure 3-10. First of all, IGF-1 receptor signaling induces activation of TRAF6 E3 ligase. In addition, LPS- or IL-1-mediated TLR/IL-1R signaling pathway promotes Akt activation through activating TRAF6, too. TRAF6 directly binds to inactive Akt in cytosol and promotes K63-linked ubiquitination of Akt. This novel modification promotes membrane recruitment, phosphorylation and activation of Akt. Activated Akt kinase promotes cell proliferation, survival and metabolism and may further induce tumorigenesis. Our findings suggest that K63-linked ubiquitination plays a crucial role in regulating activation of Akt by promoting Akt membrane recruitment, and it also involved in oncogenic Akt functionality and human cancer development.

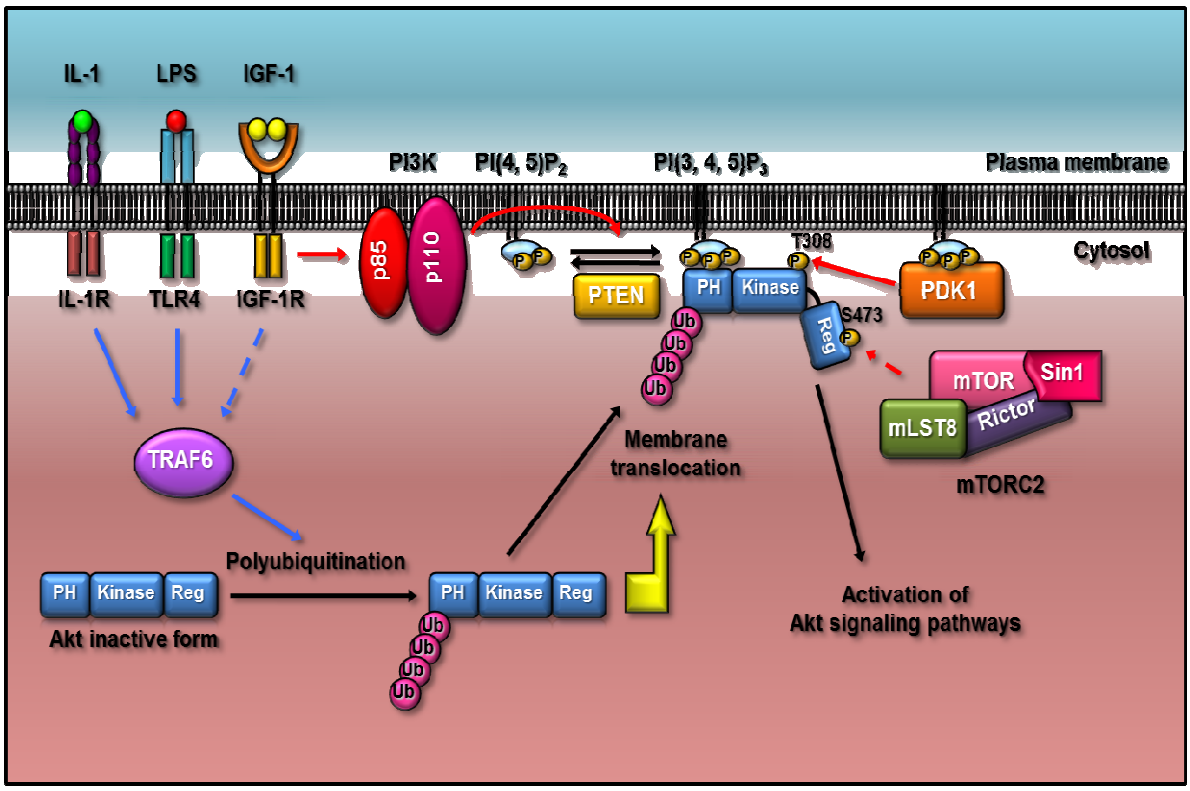

Figure 3-10. The working model for Akt activation.

IGF-1 engagement to its membrane receptor, IGF-1R, and induces TRAF6 activation. Activated TRAF6 then triggers ubiquitination of Akt, which is prerequisite to membrane recruitment of Akt, where Akt can be anchored in the membrane by binding to the PIP3, followed by phosphorylation of Akt at Thr^{308} and Ser^{473} induced by PDK1 and mTOR complex 2 (mTORC2), respectively.

(This figure was adapted from our published work in *Science, 25 (5944): 1134-1138 (2009)*; the permission was received from the journal.)

Chapter 4

Cycles of Ubiquitination and Deubiquitination Critically Regulate Growth Factor-mediated Activation of Akt Signaling

Most of this work has been published in:

W-L Yang, G. Jin, C-F Li, Y. S. Jeong, A. Moten, D. Xu, Z. Feng, W. Chen, Z. Cai, B. Darnay, W. Gu and H-K Lin **Cycles of Ubiquitination and Deubiquitination Critically Regulate Growth Factor-mediated Activation of Akt Signaling.** Science Signaling 6 (257), ra3. (2013)

RATIONALE

In the first part of my thesis study, we have discovered that Akt undergoes K63-linked ubiquitination by TRAF6 ubiquitin E3 ligase in response to growth factor or cytokine signaling stimulation. Furthermore, we have demonstrated that K63-linked ubiquitination is essential for membrane recruitment and phosphorylation of Akt and subsequent Akt signaling activation. Importantly, we found that the regulation of Akt ubiquitination is involved in human cancer development, suggesting that the regulation of Akt ubiquitination may be a key factor to determine the tumorigenicity of Akt. However, one outstanding question remains to be answered: how ubiquitination cycles of Akt are regulated in response to distinct conditions such as serum deprivation and serum stimulation. Here, we hypothesized that deubiquitinating enzymes (DUBs) in cells may serve as molecular switches for this process by sensing the concentration of growth factor in its microenvironment.

In this part of my study, the first step is to identify the DUB for deubiquitination of Akt. Furthermore, we seek to understand how this specific DUB negatively regulates growth factor-mediated membrane recruitment and activation of Akt through deubiquitination of Akt. In addition, we also want to investigate the role of ubiquitination and deubiquitination cycles in Akt

RESULTS

4-1. CYLD is a DUB for deubiquitination of Akt

To understand the mechanism by which the Akt deubiquitination process is regulated, we screened a panel of potential DUBs for Akt by performing deubiquitination assays in cells ectopically expressing DUBs. Among the DUBs, only CYLD effectively reduced the ubiquitination of Akt (Fig. 4-1A). Because Akt has three isoforms (Akt1, Akt2, and Akt3), we next examined whether CYLD displays differential specificity for various Akt isoforms and found that CYLD promoted deubiquitination of Akt1 and Akt2 (Fig. 4-1B).

CYLD inhibits activation of NF-κB and innate immune response (124, 128, 150). Mutation of the gene encoding CYLD is associated with some inherited diseases such as familial cylindromatosis, which is characterized by the development of tumors from skin appendages (cylindromatosis) (122, 124). Furthermore, loss of CYLD is found in various human cancers, suggesting that CYLD may be a tumor suppressor (151). We found that the inhibitory effect of CYLD on ubiquitination of Akt depended on its catalytic activity because the catalytically dead mutant of CYLD ($Cys^{601} \rightarrow Ala^{601}$; C601A) failed to attenuate ubiquitination of Akt (Fig. 4-1C). CYLD specifically removed K63-linked ubiquitination of Akt (Fig. 4-1D). Because TRAF6 and Skp2 are E3 ligases for Akt and play critical roles in Akt membrane recruitment, phosphorylation, and activation (152, 153), we determined whether CYLD also regulates TRAF6- or Skp2-mediated ubiquitination of Akt. Wild-type CYLD, but not its catalytic mutant, inhibited both TRAF6- and Skp2-mediated Akt ubiquitination (Fig. 4-1C and 4-1E). To determine whether CYLD is a direct DUB for Akt, we performed in vitro deubiquitination assays and found that deubiquitination of Akt was mediated by wild-type CYLD but not by the C601A mutant (Fig. 4-1F). These results suggest that CYLD is a bona fide DUB for Akt.

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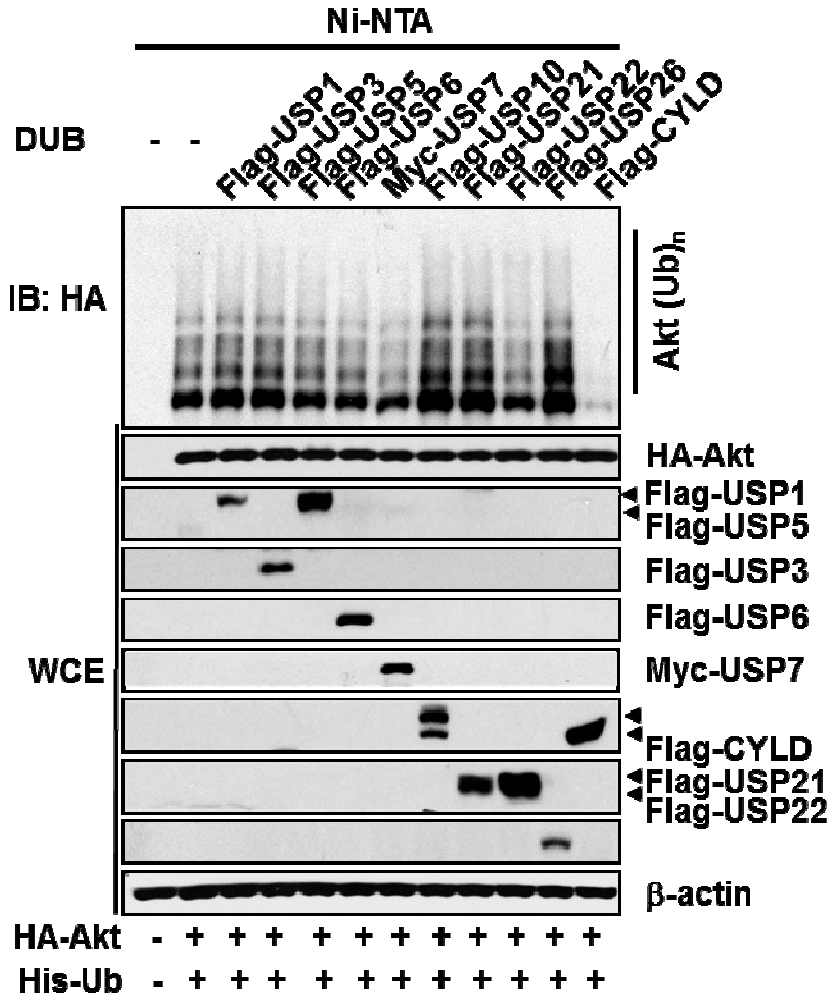

Figure 4-1. CYLD deubiquitinase promotes deubiquitination of Akt.

(A) Cellular ubiquitination assays performed in HEK293T cells transfected with His-ubiquitin (His-Ub), hemagglutinin (HA)–Akt, along with various DUB constructs. Ni–nitrilotriacetic acid (Ni-NTA), nickel bead precipitate; WCE, whole-cell extracts.

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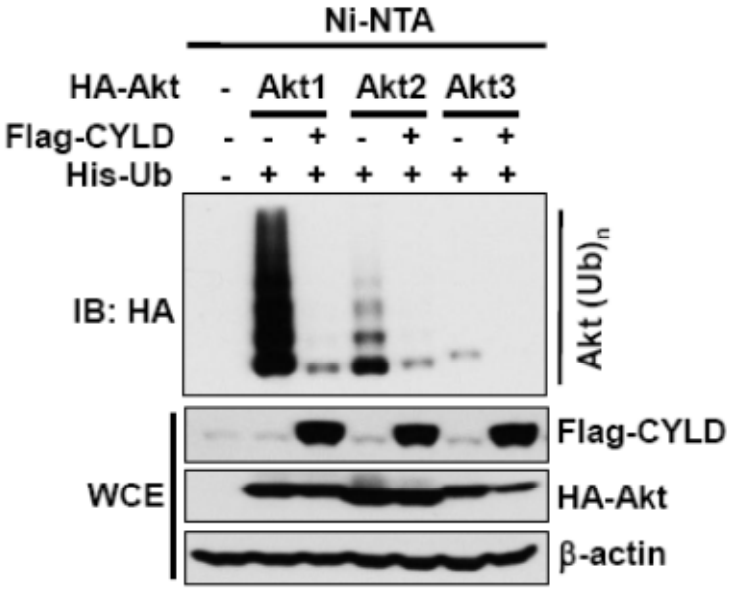

Figure 4-1. CYLD deubiquitinase promotes deubiquitination of Akt.

(B) HEK293T cells transfected with Flag-CYLD and His-Ub, along with HA-Akt1, HA-Akt2 and HA-Akt3 isoforms were lysed for *in vivo* ubiquitination assay, followed by IB analysis.

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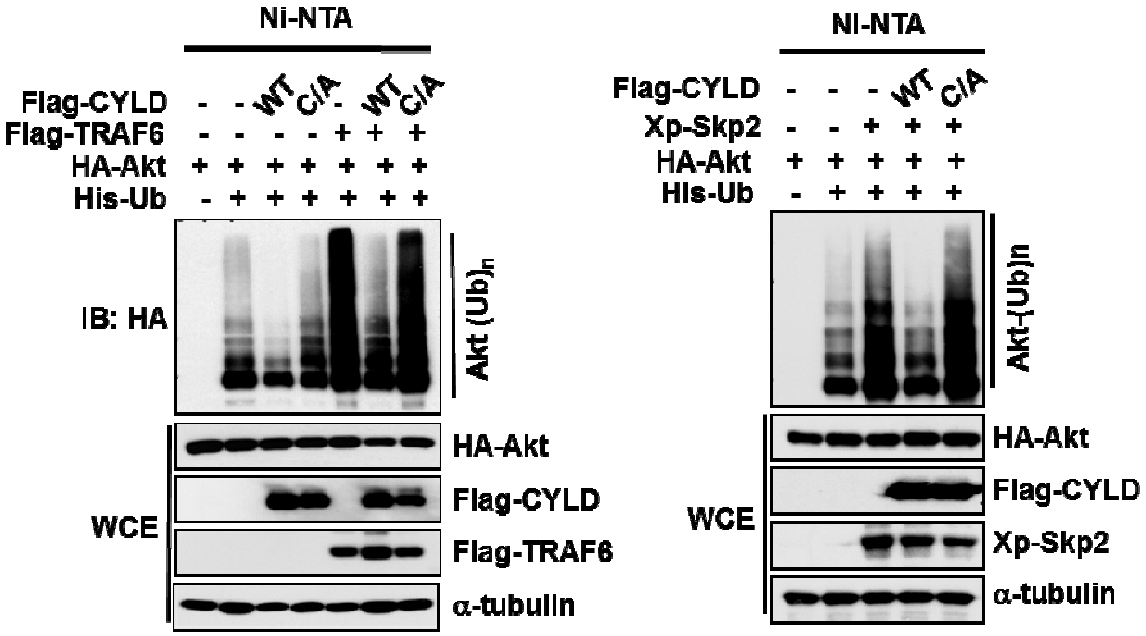

Figure 4-1. CYLD deubiquitinase promotes deubiquitination of Akt.

(C) Cellular ubiquitination assays performed in HEK293T cells transfected with HA-Akt, His-Ub, and Flag-TRAF6, along with Flag-CYLD-WT or Flag-CYLD-C/A. WT, wild type; C/A, enzyme-dead mutant (C601A).

(D) Immunoblot analysis of Akt immunoprecipitates from HEK293T cells transfected with Akt and Flag-CYLD, along with HA-Ub K48 (K48-only ubiquitin) or HA-Ub K63 (K63-only ubiquitin). IB, immunoblot; IP, immunoprecipitation.

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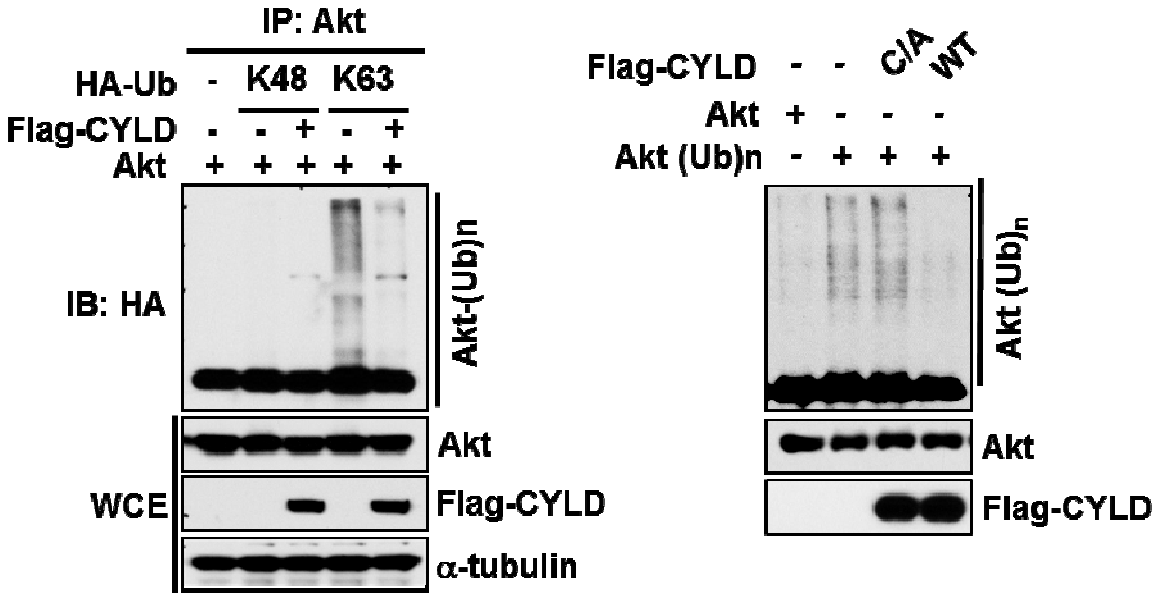

Figure 4-1. CYLD deubiquitinase promotes deubiquitination of Akt.

(E) Cellular ubiquitination assays performed in HEK293T cells transfected with HA-Akt, His-Ub, and Xp-Skp2, along with Flag-CYLD-WT or Flag-CYLD-C/A.

(F) In vitro deubiquitination assays with purified ubiquitinated Akt proteins incubated with purified Flag-CYLD-C/A or Flag-CYLD-WT proteins.

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4-2. CLYD interacts with Akt and its deficiency induces basal Akt ubiquitination.

We next determined whether CYLD deficiency promotes ubiquitination of endogenous Akt in response to IGF-1 stimulation. In wild-type primary mouse embryonic fibroblasts (MEFs), endogenous Akt was basally ubiquitinated under serum-starved conditions and ubiquitination was increased by IGF-1, consistent with our previous report (Fig. 4-2A) (152). Ubiquitination of endogenous Akt under serum-starved conditions in *Cyld*$^{-/-}$ MEFs was similar to that seen in wild-type MEFs treated with IGF-1, but was not further increased by IGF-1 treatment (Fig. 4-2A). Similarly, prostate cancer cells lacking CYLD also displayed increased basal ubiquitination of Akt in the absence of IGF-1 treatment (Fig. 4-2B). These results suggest that CYLD keeps Akt in the hypoubiquitinated state and that its deficiency facilitates ubiquitination of Akt.

We next determined whether Akt associates with CYLD. Co-immunoprecipitation assays showed that exogenously expressed Akt1 interacted with both wild-type and mutant CYLD (C601A) (Fig. 4-2C). Likewise, exogenously expressed Akt2 and Akt3 also interacted with CYLD (data not shown). Notably, phosphorylation of Akt at Thr^{308} and Ser^{473}, which indicate activation of Akt, was attenuated by wild-type CYLD but not by C601A CYLD (Fig. 4-2C). Moreover, we found that endogenous Akt interacted with CYLD under serum-starved conditions in a reciprocal immunoprecipitation assay (Fig. 4-2D). The interaction between CYLD and Akt was lost after 15 min of IGF-1 treatment (Fig. 4-2E), whereas the interaction between Akt and TRAF6 was induced at the same time point (152), suggesting that CYLD and TRAF6 may compete with each other for Akt binding. Indeed, we found that TRAF6 overexpression inhibited the binding of CYLD to Akt in a dose-dependent manner (Fig. 4-2F). These results suggest that CYLD interacts with Akt under serum-starved conditions and dissociates from Akt upon growth factor stimulation, which may allow E3 ligases to bind to and ubiquitinate Akt.

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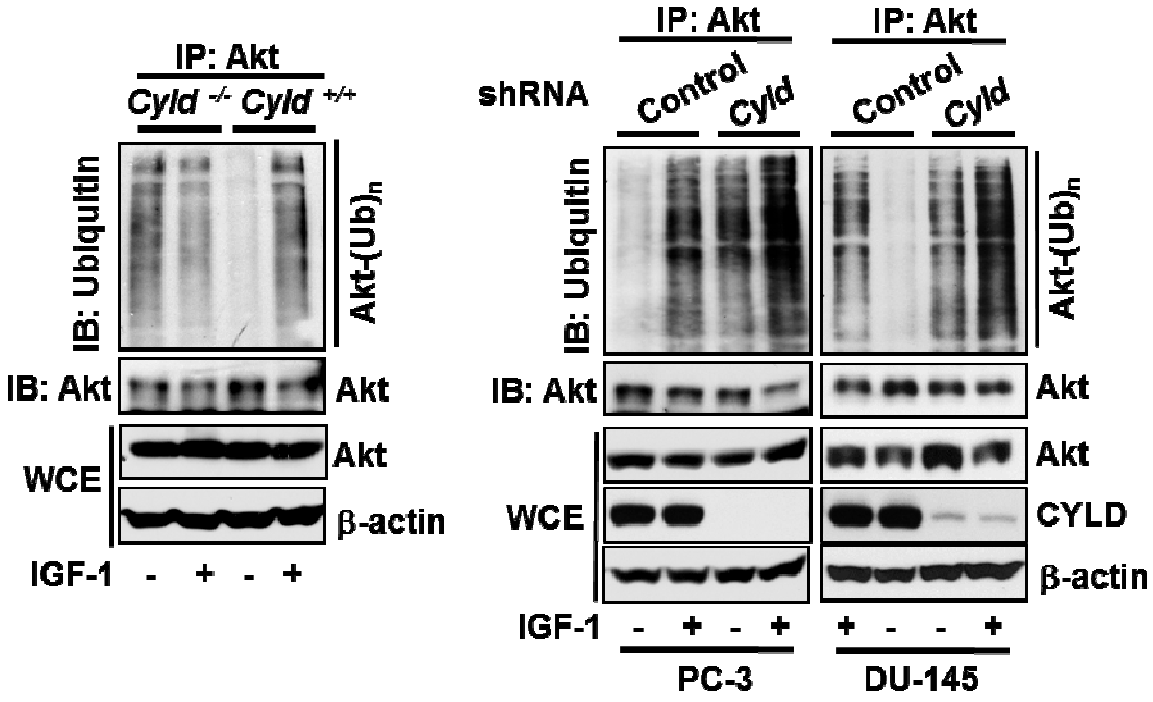

Figure 4-2. CYLD interacts with Akt and inhibits the ubiquitination of endogenous Akt.

(A) Immunoblot analysis of Akt immunoprecipitates from *Cyld*$^{+/+}$ and *Cyld*$^{-/-}$ MEFs that were serum starved and treated with or without IGF-1.

(B) Immunoblot analysis of Akt immunoprecipitates from control or CYLD stable knockdown PC-3 or DU-145 cells that were serum starved and treated with or without IGF-1.

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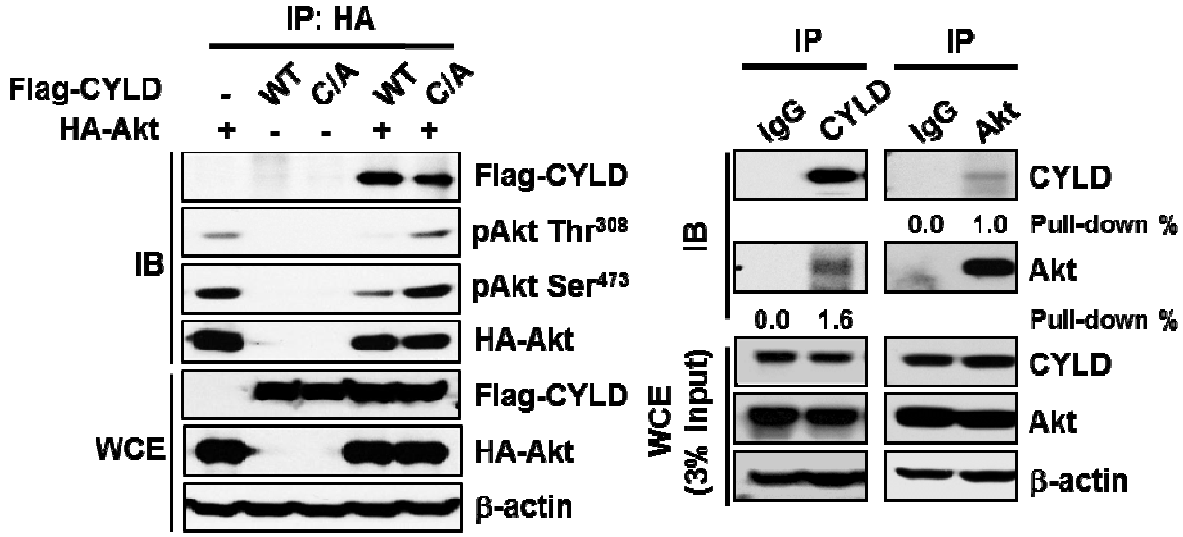

Figure 4-2. CYLD interacts with Akt and inhibits the ubiquitination of endogenous Akt.

(C) Immunoblot analysis of HA immunoprecipitates from HEK293T cells transfected with HA-Akt along with Flag-CYLD-WT or Flag-CYLD-C/A.

(D) Immunoblot analysis of CYLD or Akt immunoprecipitates from serum-starved PC-3 cells. (B) PC-3 cells were serum-starved and harvested for co-immunoprecipitation assay with CYLD or Akt antibody respectively, followed by IB analysis. Pull down % indicates percentage of Akt or CYLD protein pulled down from total Akt or CYLD protein.

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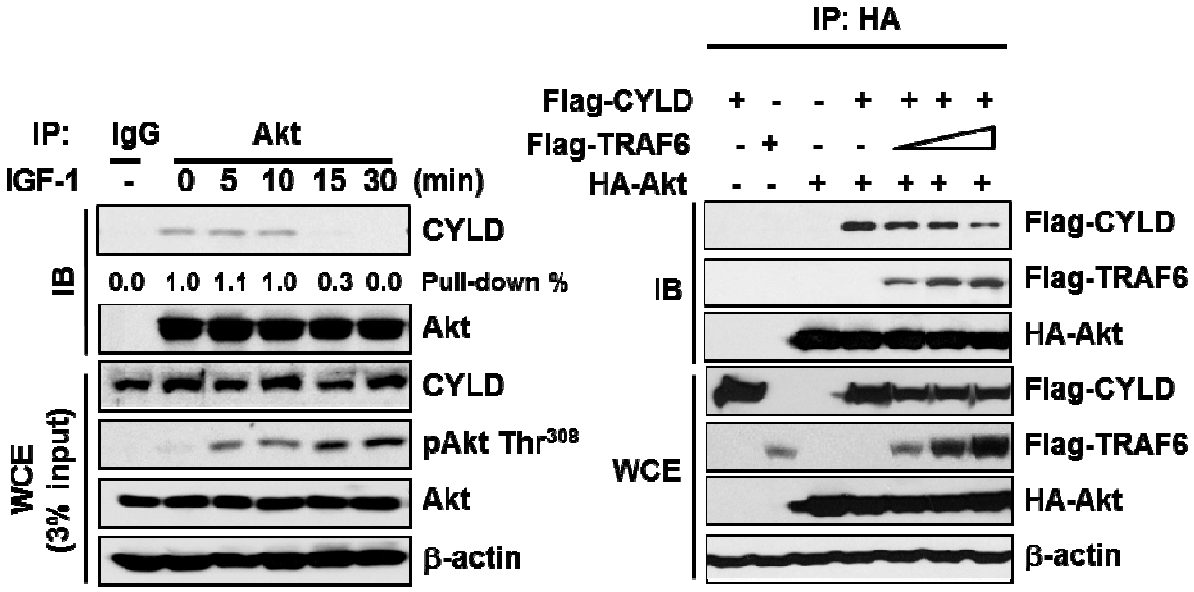

Figure 4-2. CYLD interacts with Akt and inhibits the ubiquitination of endogenous Akt.

(E) Immunoblot analysis of Akt immunoprecipitates from PC-3 cells that were serum-starved and treated with IGF-1 at various time points.

(F) Immunoblot analysis of HA immunoprecipitates from HEK293T cells transfected with HA-Akt along with Flag- CYLD or various amounts of Flag-TRAF6.

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4-3. The interaction of Akt with CYLD is independent of its ubiquitination or phosphorylation status

We next examined whether the ubiquitination status of Akt affects its interaction with CYLD. CYLD bound to both non-ubiquitinated and ubiquitinated Akt forms (Fig. 4-3A), and both wild-type Akt and the ubiquitination-deficient K8R mutant (152) interacted with CYLD (Fig. 4-3B). Because IGF-1 disrupts the interaction between Akt and CYLD, we also determined whether phosphorylation of Akt attenuated (or disrupted) its interaction with CYLD. Both wild-type and the constitutively active Akt mutant T308D/S473D (Akt-DD) interacted with CYLD (Fig. 4-3C), suggesting that the phosphorylation of Akt does not affect Akt and CYLD interaction. Consistent with this notion, the phosphorylation-defective Akt mutant T308A/T450A/S473A (Akt-AAA) also bound to CYLD, and overexpression of TRAF6 induced a dose-dependent decrease in the interaction between CYLD and the Akt-AAA mutant (Fig. 4-3D). These results suggest that the ubiquitination or phosphorylation status of Akt may not be critical for its interaction with CYLD.

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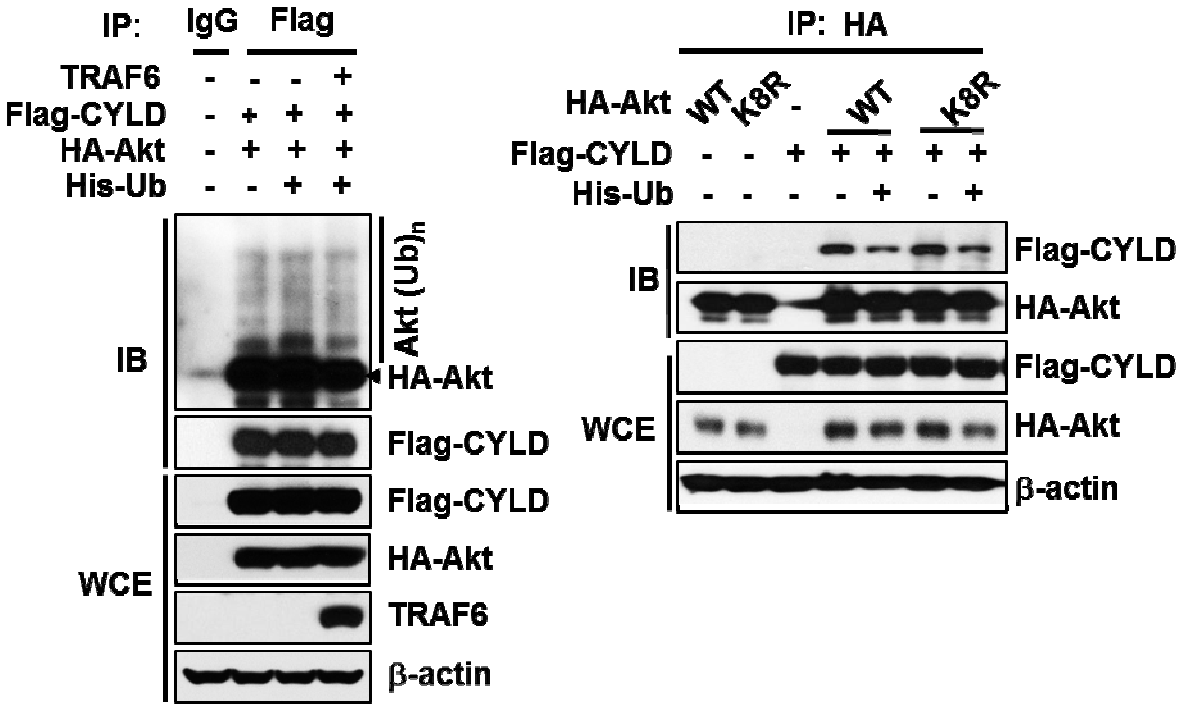

Figure 4-3. Ubiquitination and phosphorylation status of Akt does not affect the interaction of Akt with CYLD.

(A) Immunoblot analysis of Flag immunoprecipitates from HEK293T cells transfected with Flag-CYLD, HA-Akt, His-Ub, and TRAF6.

(B) Immunoblot analysis of HA immunoprecipitates from HEK293T cells transfected with Flag-CYLD and His-Ub, along with HA-Akt-WT or HA-Akt K8R.

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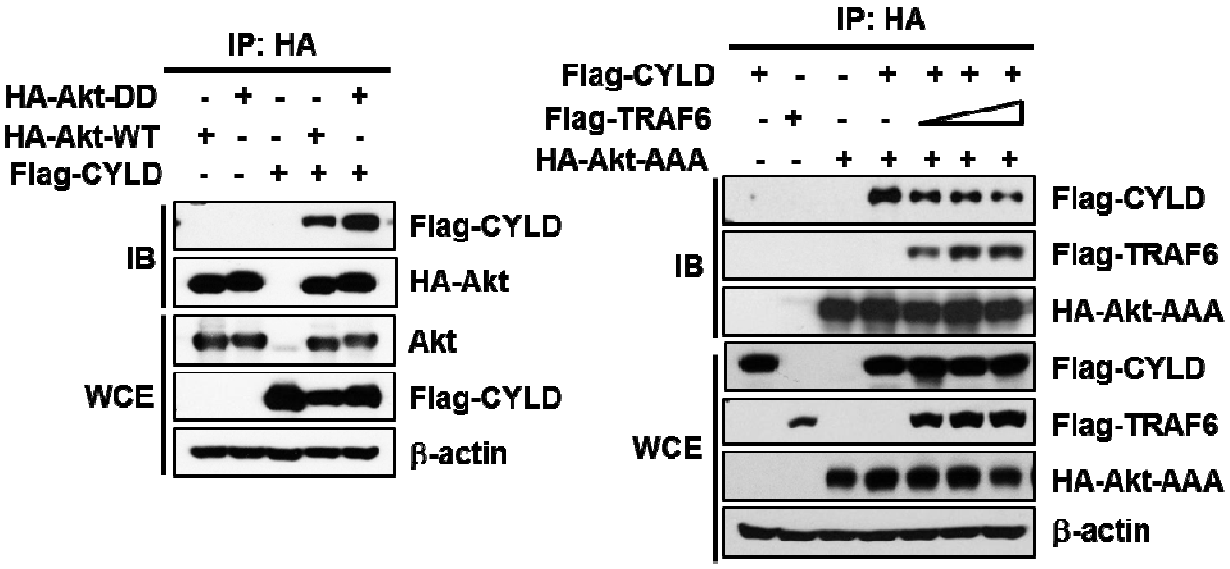

Figure 4-3. Ubiquitination and phosphorylation status of Akt does not affect the interaction of Akt with CYLD.

(A) HEK293T cells transfected with Flag-CYLD, along with HA-Akt-WT or HA-Akt-DD (T308D/S473D) were harvested for immunoprecipitation with HA antibody, followed by IB analysis.

(B) HEK293T cell transfected with Flag-CYLD and HA-Akt-AAA (T308A/T450A/ S473A), along with dose-dependent Flag-TRAF6 were harvested for immunoprecipitation with HA antibody, followed by IB analysis.

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4-4. Ubiquitination and deubiquitination of Akt are independent of PI3K activity.

PI3K activity plays an important role in growth facto-induced activation of Akt. To determine whether PI3Kis involved in ubiquitination and deubiquitination of Akt, we performed cellular ubiquitination and deubiquitination assays for endogenous Akt in the presence or absence of the PI3K inhibitor LY294002. Inhibition of PI3K activity did not inhibit IGF-1-induced ubiquitination and CYLD-mediated deubiquitination of Akt (Fig. 4-4A and 4-4B).

We further determined whether polyubiquitination-mediated membrane recruitment of Akt could lead to its activation without PI3K activity. To this end, human embryonic kidney (HEK) 293T cells were cotransfected with wild-type Akt, ubiquitination-deficient K8R Akt mutant, or cancer-associated E17K Akt mutant, which displays much higher ubiquitination of Akt compared to wild-type Akt (152), in the presence or absence of LY294002. Consistent with our previous observation (152), we found that phosphorylation of Akt was increased in cells expressing the E17K mutant, whereas those expressing the ubiquitination-deficient K8R mutant showed reduced phosphorylation of Akt compared to wild-type Akt. However, PI3K inhibition by LY294002 reduced phosphorylation of wild-type Akt and the E17K and K8R mutants (Fig. 4-4C), suggesting that although polyubiquitination of Akt is required for its phosphorylation and activation, it is not sufficient to lead to Akt activation without PI3K activation.

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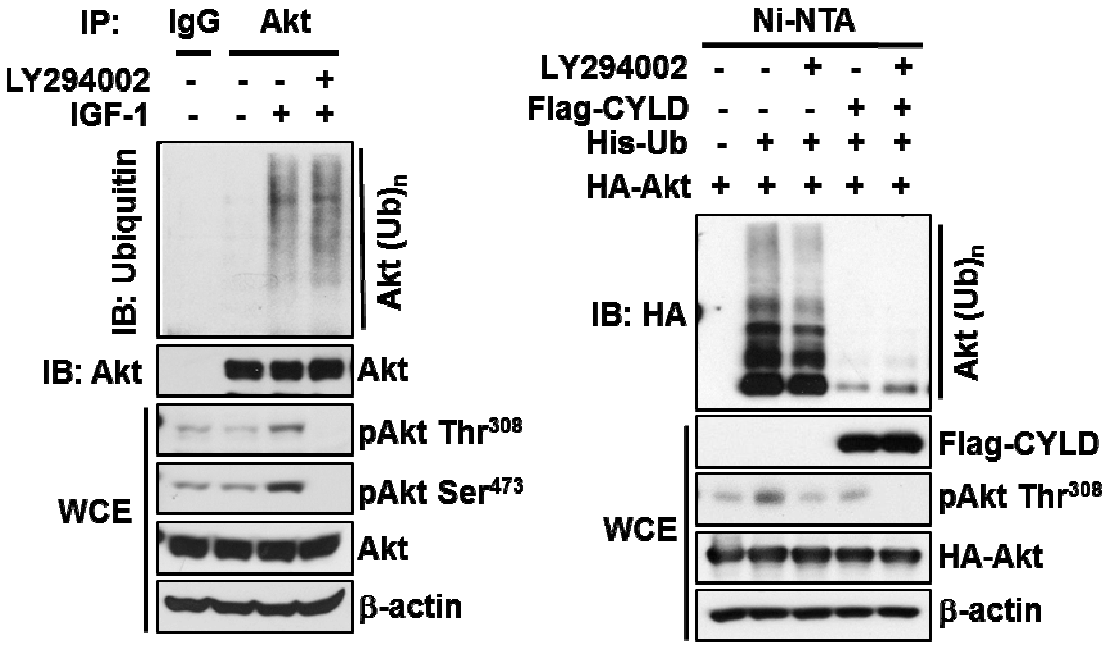

Figure 4-4. PI3K activity is not required for ubiquitination and deubiquitination of Akt.

(A) Immunoblot analysis of HA immunoprecipitates from serum-starved PC-3 cells that were pretreated with or without the PI3K inhibitor LY294002 and treated with or without IGF-1.

(B) Cellular ubiquitination assays performed in HEK293T cells transfected with HA-Akt, His-Ub, and Flag-CYLD and pretreated with or without LY294002.

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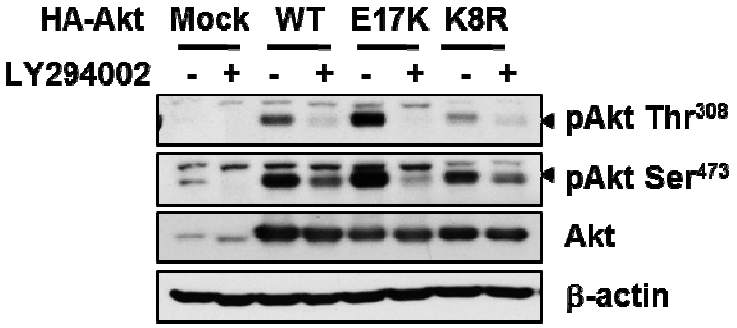

Figure 4-4. PI3K activity is not required for ubiquitination and deubiquitination of Akt.

(C) Immunoblot analysis of HEK293T cells transfected with HA-Akt-WT, HA-Akt-E17K, or HA-Akt-K8R and treated with or without LY294002.

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4-5. CYLD deficiency promotes membrane recruitment and phosphorylation of Akt.

The finding that CYLD interacts with Akt and suppresses ubiquitination of Akt prompted us to determine whether CYLD prevents phosphorylation of Akt in response to growth factor stimulation. Indeed, *Cyld*$^{-/-}$ MEFs displayed higher basal phosphorylation of Akt (at 0 min) and slightly enhanced IGF-1-mediated phosphorylation of Akt compared to wild-type MEFs (Fig. 4-5A), which correlated with the increased basal ubiquitination of Akt caused by Cyld deficiency (Fig. 4-2A). Likewise, CYLD knockdown also promoted basal and IGF-1-and EGF-induced phosphorylation of Akt in prostate cancer cell lines (Fig. 4-5B and 4-5C). Conversely, restoration of wild-type CYLD, but not the C601A mutant, into CYLD knockdown cancer cells reversed IGF-1-induced phosphorylation of Akt, suggesting that the enzymatic activity of CYLD plays a critical role in suppressing growth factor-induced phosphorylation of Akt (Fig. 4-5D). Accordingly, our results suggest that CYLD opposes ubiquitination of Akt and thereby inhibits its phosphorylation in response to stimulation with growth factors.

We have shown that ubiquitination of Akt is critical for growth factor-mediated membrane recruitment and activation of Akt (152). Given that CYLD opposes ubiquitination of Akt, it is possible that CYLD prevents membrane recruitment of Akt, in turn inhibiting its phosphorylation and activation. To test this hypothesis, we performed biochemical fractionation experiments to determine whether CYLD regulates membrane recruitment of Akt in the presence or absence of IGF-1 and EGF treatment. Consistent with this notion, either Cyld deficiency or CYLD knockdown enhanced basal membrane recruitment of Akt as well as basal and IGF-1- and EGF-mediated phosphorylation of Akt in the plasma membrane (Fig. 4-5E, 4-5F and 4-5G). Similar results were also obtained from immunofluorescence analysis of wild-type and *Cyld*$^{-/-}$ MEFs (Fig. 4-5H). We next determined whether PI3K activity is required for membrane translocation of Akt in wild-type and *Cyld*$^{-/-}$ MEFs. PI3K inhibition efficiently blocked IGF-1-induced membrane translocation of Akt in both wild-type and *Cyld*$^{-/-}$MEFs but failed to inhibit constitutive membrane recruitment of Akt in *Cyld*$^{-/-}$ MEFs under serum-starved conditions (Fig. 4-5I). Thus, our results support the notion that CYLD opposes Akt ubiquitination, in turn inhibiting growth factor-mediated membrane recruitment and phosphorylation of Akt.

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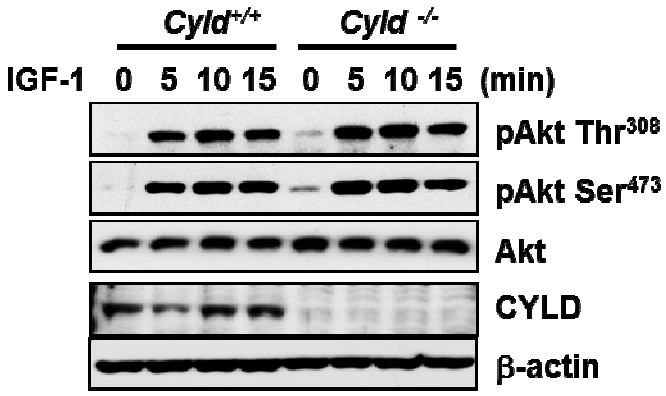

Figure 4-5. CYLD deficiency facilitates growth factor–mediated membrane recruitment and phosphorylation of Akt.

(A) Immunoblot analysis of *Cyld*$^{+/+}$ and *Cyld*$^{-/-}$ MEFs that were serum-starved and treated with IGF-1 at various time points.

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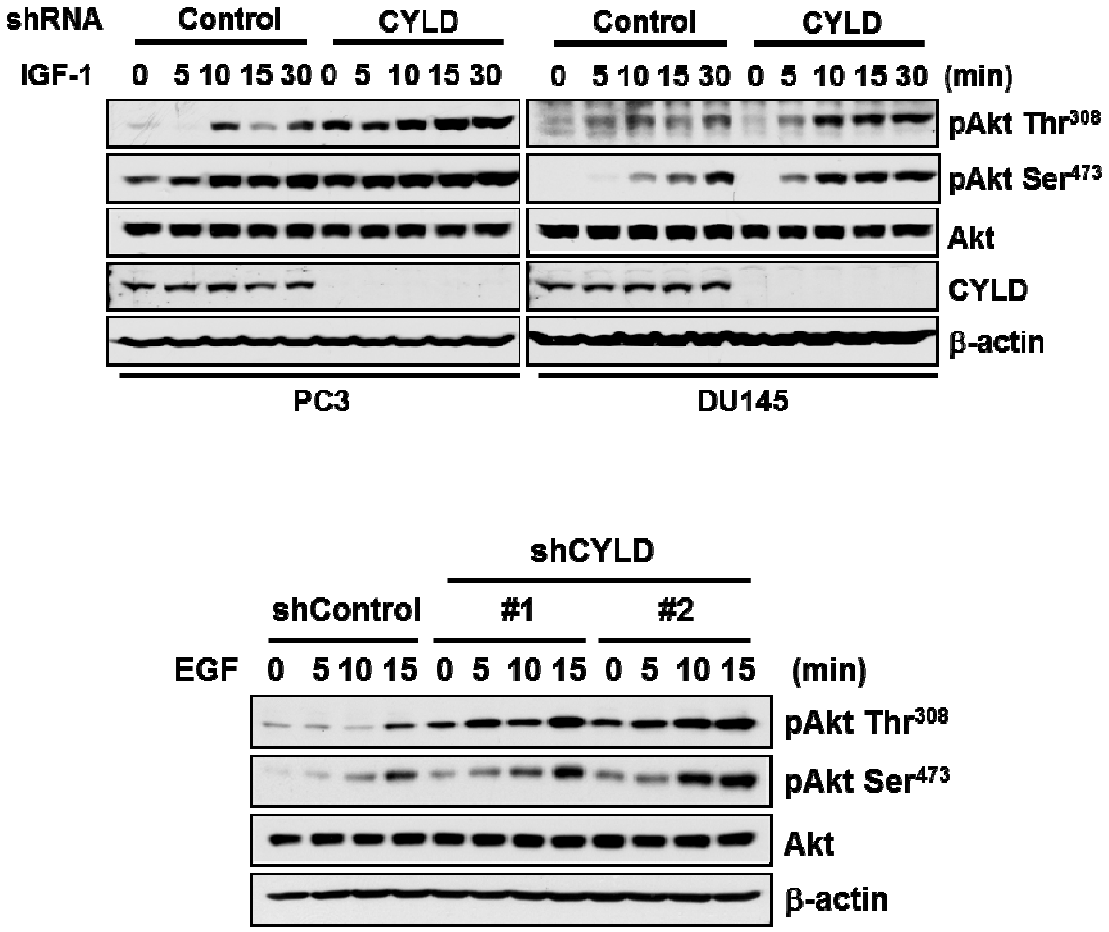

Figure 4-5. CYLD deficiency facilitates growth factor–mediated membrane recruitment and phosphorylation of Akt.

(B) Immunoblot analysis of control or CYLD stable knockdown PC-3 or DU-145 cells that were serum-starved and treated with IGF-1 at various time points.

(C) Immunoblot analysis of control or two different CYLD-knockdown PC-3 cell lines that were serum-starved and treated with EGF at various time points.

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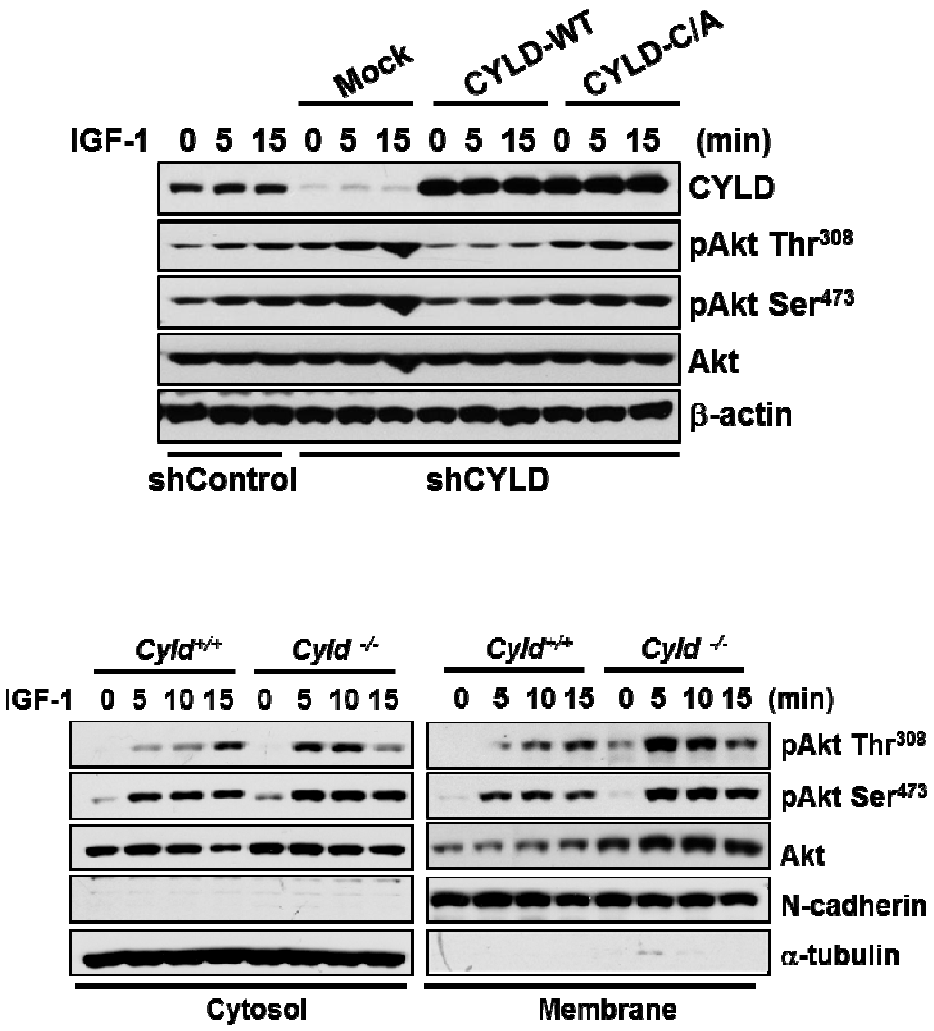

Figure 4-5. CYLD deficiency facilitates growth factor–mediated membrane recruitment and phosphorylation of Akt.

(D) Immunoblot analysis of CYLD-knockdown PC-3 cells that were transfected with mock, Flag-CYLD-WT, or Flag-CYLD-C/A, serum-starved, and treated with IGF-1 at various time points.

(E) Immunoblot analysis of membrane and cytosolic fractions of $Cyld^{+/+}$ and $Cyld^{-/-}$MEFs that were serum-starved and treated with IGF-1 at various time points.

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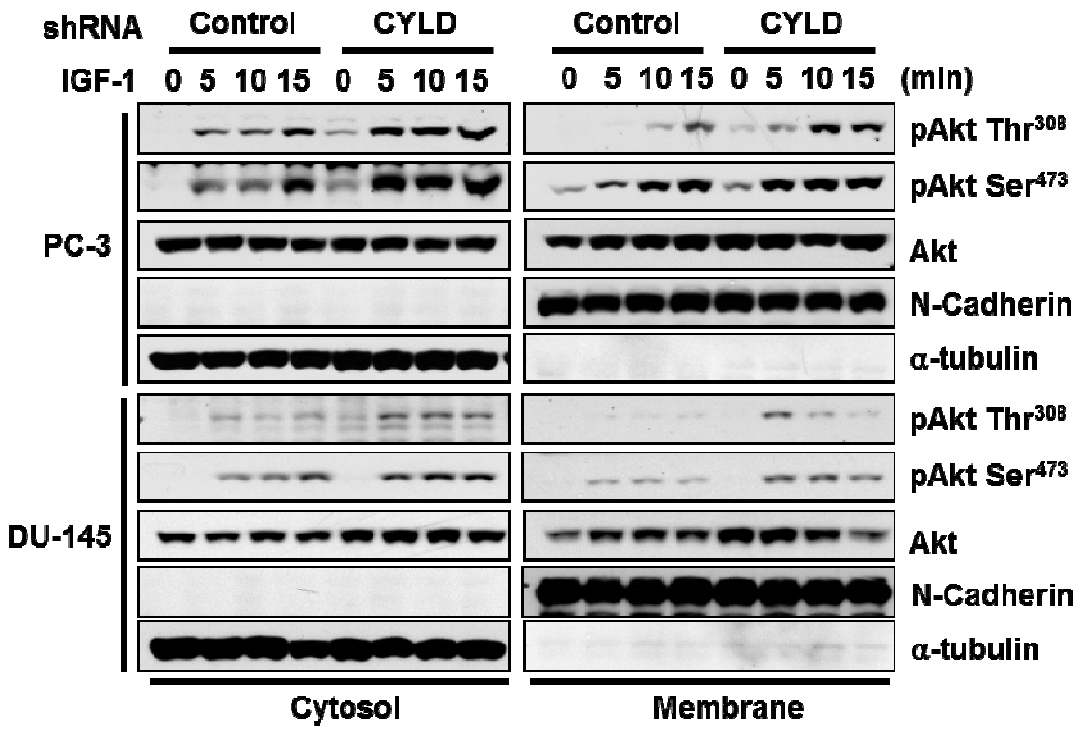

Figure 4-5. CYLD deficiency facilitates growth factor–mediated membrane recruitment and phosphorylation of Akt.

(F) Immunoblot analysis of membrane and cytosolic fractions of control or CYLD stable knockdown PC-3 or DU-145 cells that were serum-starved and treated with IGF-1 at various time points.

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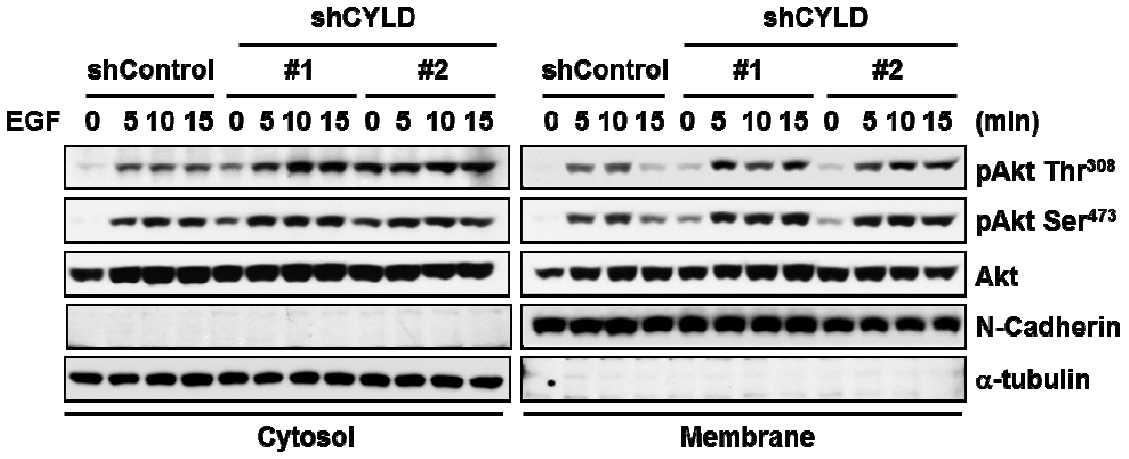

Figure 4-5. CYLD deficiency facilitates growth factor–mediated membrane recruitment and phosphorylation of Akt.

(G) Immunoblot analysis of membrane and cytosolic fractions of control or two CYLD-knockdown PC-3 cell lines that were serum-starved and treated with EGF at various time points.

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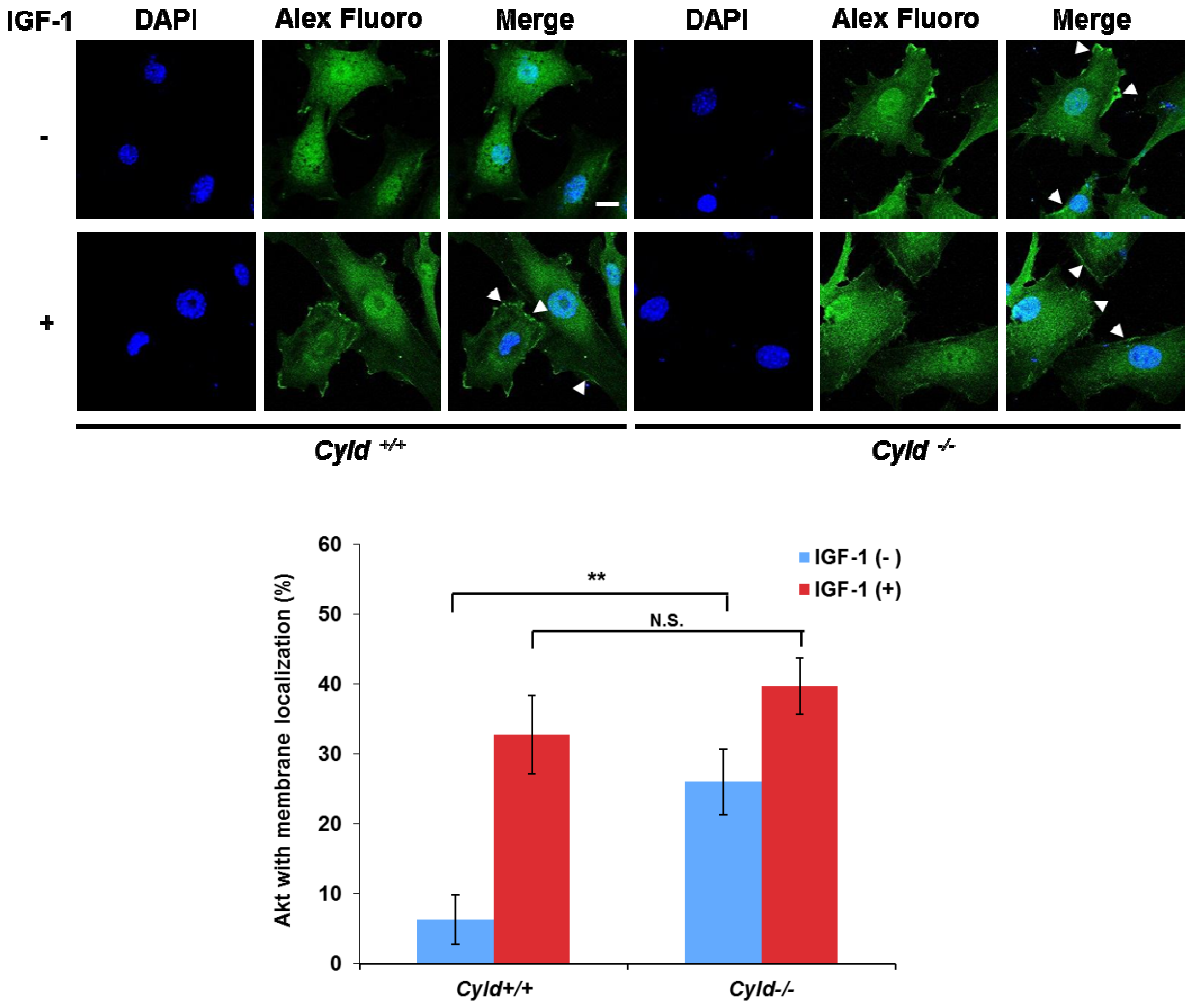

Figure 4-5. CYLD deficiency facilitates growth factor–mediated membrane recruitment and phosphorylation of Akt.

(H) wild-type and Cyld$^{-/-}$ MEFs were serum-starved, treated with/without IGF-1, and fixed for immunofluorescence. The arrowhead indicates the membrane localization of Akt. The scale bar represents 10 μm. The quantification result is shown in lower panel. The results is presented as mean percentage values from three biological replicates; N.S., non-significant and ***P < 0.001 for all pairwise comparisons by Pearson chi-square test.

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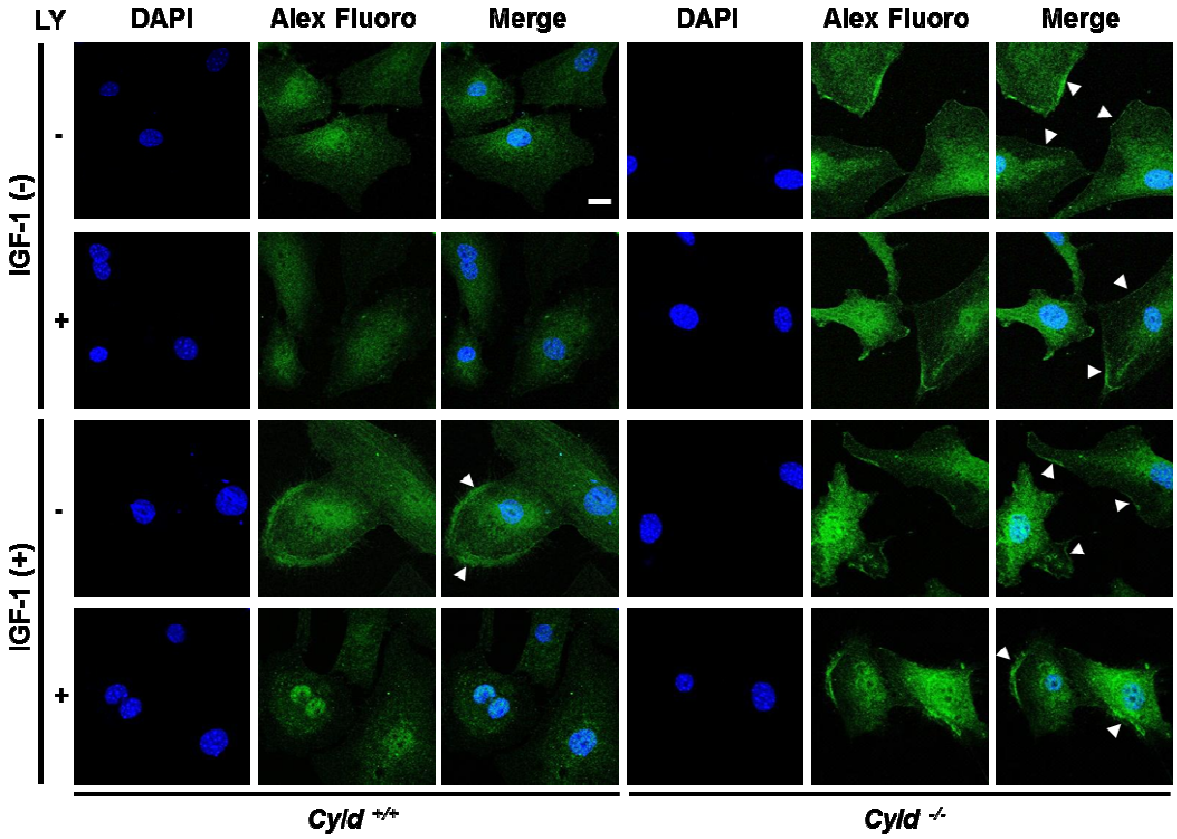
LY
DAPI
Alex Fluoro
Merge
DAPI
Alex Fluoro
Merge
IGF-1 (-)
-
+
IGF-1 (+)
-
+
Cyld +/+
Cyld -/-

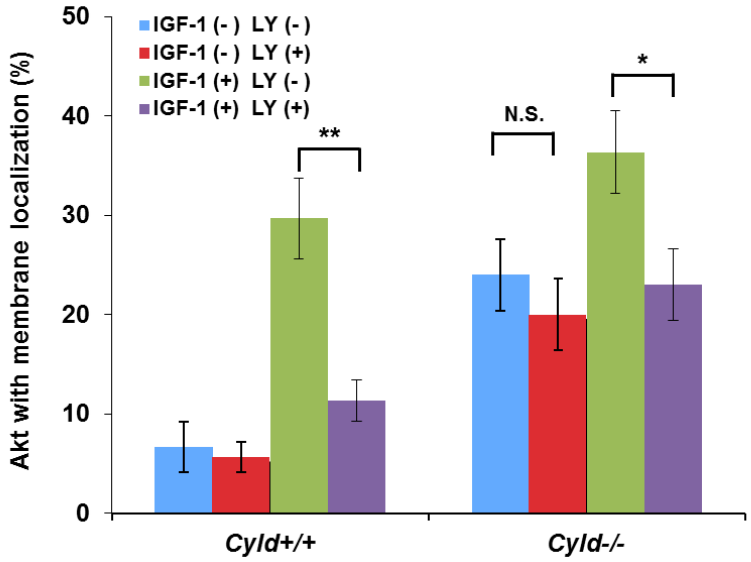
IGF-1 (-) LY (-)
IGF-1 (-) LY (+)
IGF-1 (+) LY (-)
IGF-1 (+) LY (+)
Akt with membrane localization (%)
50
40
30
20
10
0
**
N.S.
*
Cyld+/+
Cyld-/-

Figure 4-5. CYLD deficiency facilitates growth factor–mediated membrane recruitment and phosphorylation of Akt.

(I) Wild-type and *Cyld*$^{-/-}$ MEFs were serum-starved and pretreated with or without LY294002 (LY). Then cells were treated with or without IGF-1 and fixed for immunofluorescence. The arrowhead indicates the membrane localization of Akt. The scale bar represents 10 μm. The quantification result is shown in lower panel. The result is presented as mean percentage values from three biological replicates; N.S., non-significant and *P < 0.05, ***P < 0.001 for all pairwise comparisons by Pearson chi-square test.

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4-6. CYLD deficiency promotes cancer cell proliferation, cell survival, and tumorigenesis.

Akt plays a crucial role in various biological processes, such as cell proliferation, cell survival, and metabolism, and thus in tumorigenesis. Because CYLD suppresses ubiquitination and activation of Akt, it is possible that CYLD may also inhibit cancer cell proliferation and survival. In support of this notion, we found that prostate cancer cells with CYLD knockdown proliferated faster than control knockdown cancer cells (Fig. 4-6A). In addition, CYLD-deficient prostate cancer cells showed enhanced cell survival in response to treatment with the apoptosis inducer cisplatin (Fig. 4-6B).

To determine whether CYLD inhibits cell proliferation and survival by suppressing activation of Akt, we performed cell proliferation and apoptosis assays on control and CYLD-knockdown prostate cancer cells treated with or without the PI3K inhibitors LY294002 or wortmannin. PI3K inhibitors compromised the increase in cell proliferation and survival in CYLD deficient prostate cancer cells, suggesting that CYLD inhibits these biological events through the PI3K-Akt pathway (Fig. 4-6C and 4-6D). However, because the PI3K inhibitors did not completely abolish the effect of CYLD deficiency on cell proliferation and survival, we cannot exclude the possibility that CYLD may also regulate other pathways. Because CYLD inhibits NF-κB activation (122, 128, 150), it is probable that both PI3K-Akt and NF-κB signaling pathways are involved in CYLD-regulated cell proliferation and survival.

Akt promotes glucose uptake for glycolysis and adenosine triphosphate production by inducing the plasma membrane localization of glucose transporters, such as Glut1 and Glut4 (154-157). Because CYLD attenuates activation of Akt, we hypothesized that CYLD may inhibit membrane translocation of both Glut1 and Glut4 and glucose uptake. Indeed, we found that *Cyld*$^{-/-}$ MEFs showed increased membrane localization of Glut1 and Glut4 compared to wild-type MEFs (Fig. 4-6E). Although glucose uptake in *Cyld*$^{-/-}$ MEFs was slightly higher than that in wild-type MEFs, this difference was not statistically significant (Fig. 4-6F). However, CYLD-knockdown prostate cancer cells displayed increased membrane localization of Glut1, but not of Glut4, and higher glucose uptake compared to control knockdown cells (Fig. 4-6E and 4-6F). Accordingly, these results suggest that CYLD inhibits proliferation, survival, membrane translocation of Glut1, and glucose uptake of prostate cancer cells.

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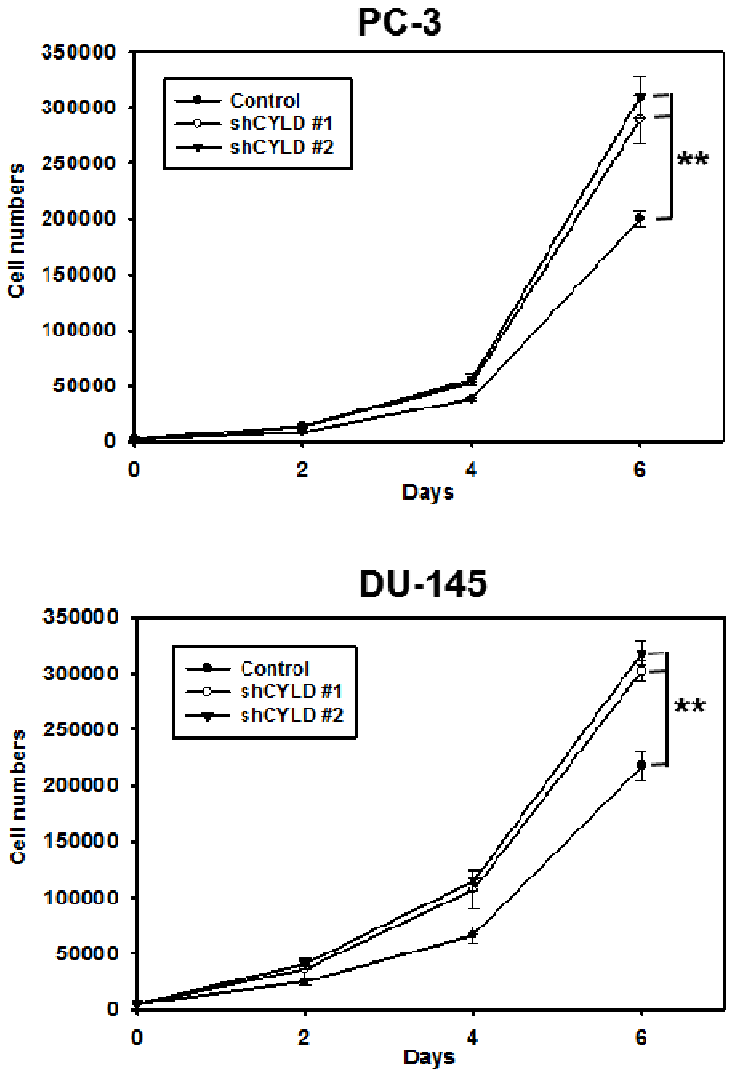

Figure 4-6. CYLD inhibits cancer cell proliferation, cell survival, and glucose uptake.

(A) Cell proliferation in control or CYLD stable knockdown PC-3 or DU-145 cells, presented as means ± SD from three biological replicates. **P < 0.01, ***P <0.001 for all pairwise comparisons by one-way analysis of variance (ANOVA) and post hoc intergroup comparisons with Sidak test.

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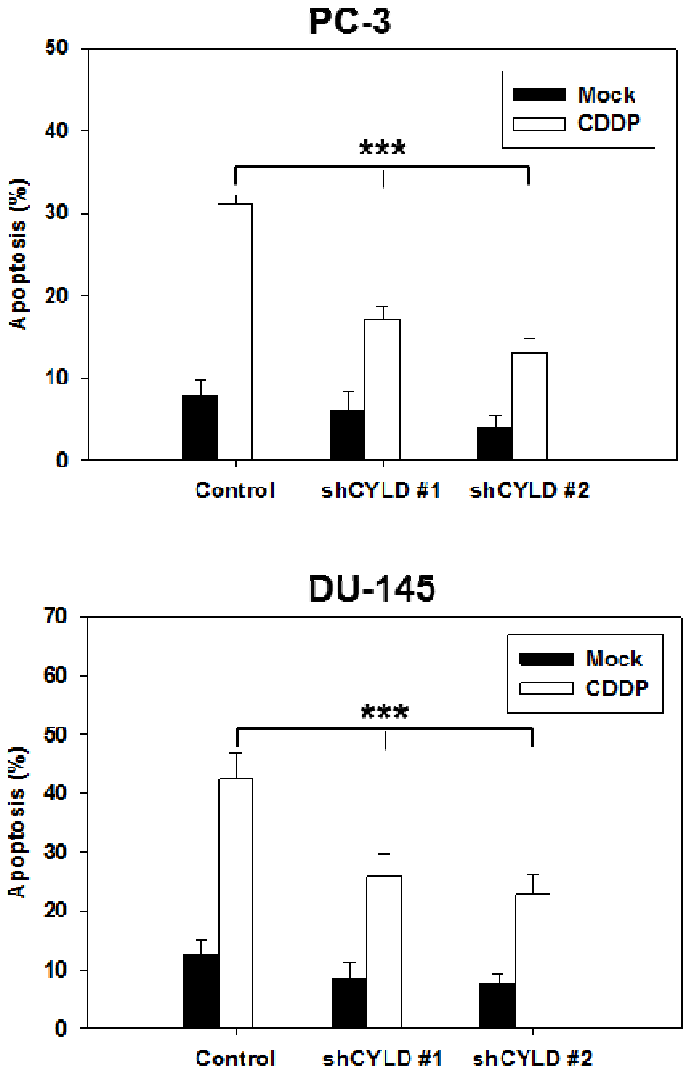

Figure 4-6. CYLD inhibits cancer cell proliferation, cell survival, and glucose uptake.

(B) Cell apoptosis as determined by annexin V staining and flow cytometric analysis in control or CYLD stable knockdown PC-3 or DU-145 cells that were treated with vehicle or cisplatin. Results are presented as mean percentages from three biological replicates. *P < 0.05, **P < 0.01 for all pairwise comparisons by Pearson χ^2 test.

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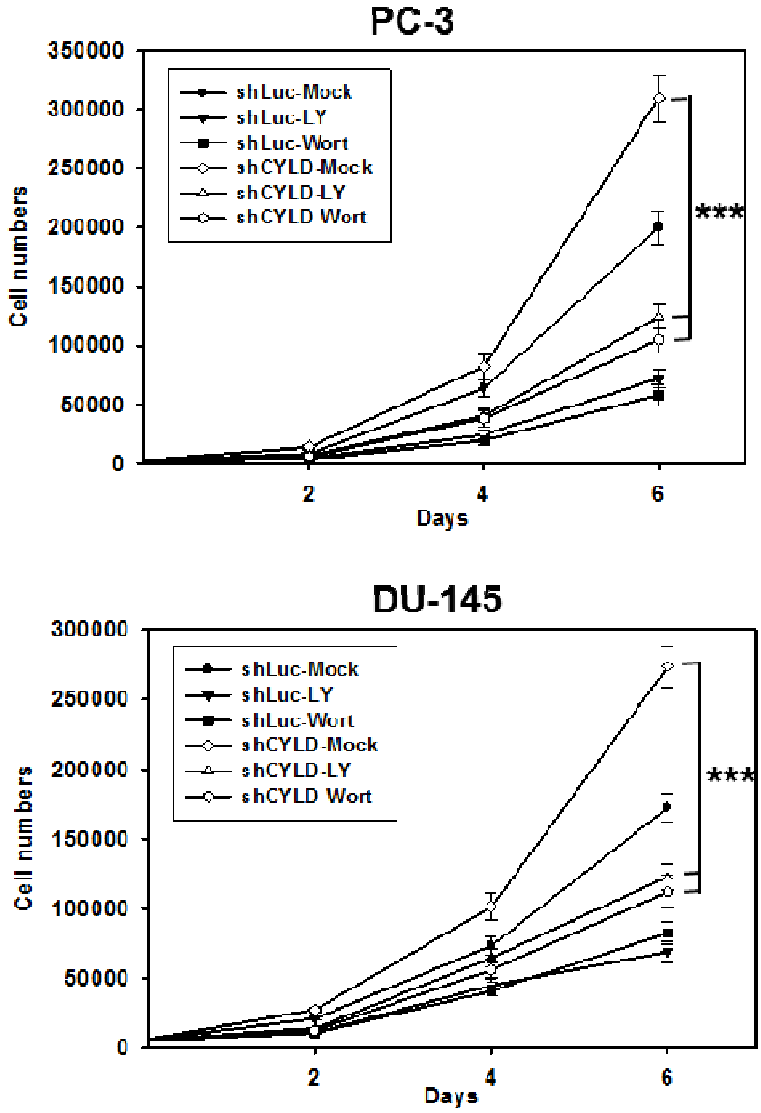

Figure 4-6. CYLD inhibits cancer cell proliferation, cell survival, and glucose uptake.

(C) Cell proliferation in control or CYLD-knockdown PC-3 or DU-145 cells treated with vehicle, LY294002 (LY), or wortmannin (Wort), presented as means ± SD from three biological replicates. ***P < 0.001 for all pairwise comparisons by one-way ANOVA and post hoc intergroup comparisons with Sidak test.

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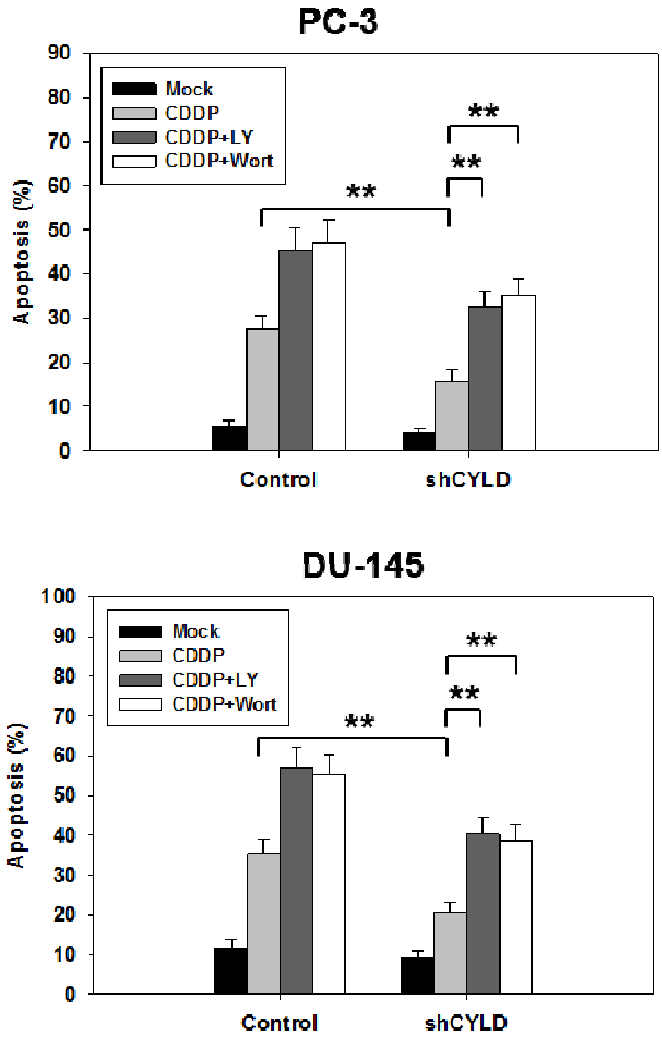

Figure 4-6. CYLD inhibits cancer cell proliferation, cell survival, and glucose uptake.

(D) Cell apoptosis as determined by annexin V staining and flow cytometric analysis in control or CYLD stable knockdown PC-3 or DU-145 cells that were pretreated with vehicle, LY294002 (LY), or wortmannin (Wort), then with vehicle or cisplatin. Results are presented as mean percentages from three biological replicates. *P < 0.05, **P < 0.01 for all pairwise comparisons by Pearson χ^2 test.

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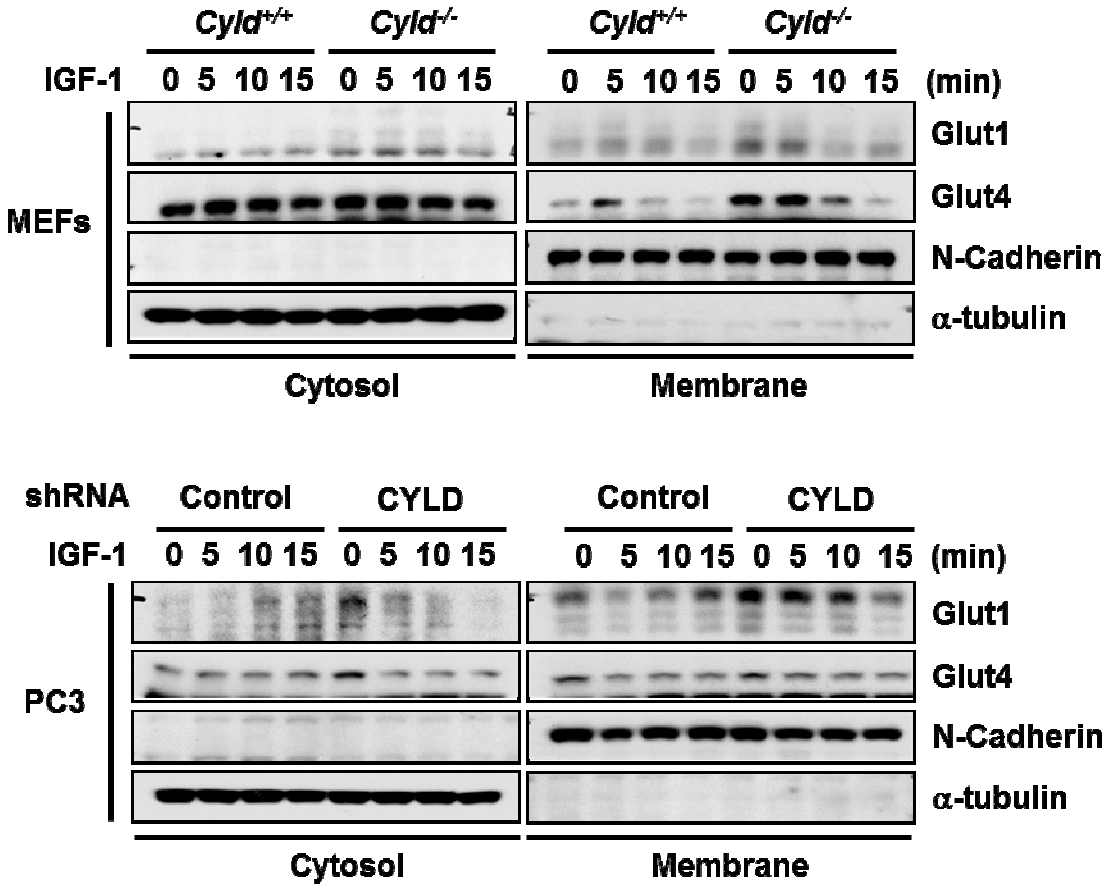

Figure 4-6. CYLD inhibits cancer cell proliferation, cell survival, and glucose uptake.

(E) Immunoblot analysis ofGlut1 and Glut4 in cytosolic or membrane fractions of *Cyld*$^{+/+}$ and *Cyld*$^{-/-}$ MEFs or PC-3 cells with control or CYLD knockdown that were treated with IGF-1 for the indicated time intervals.

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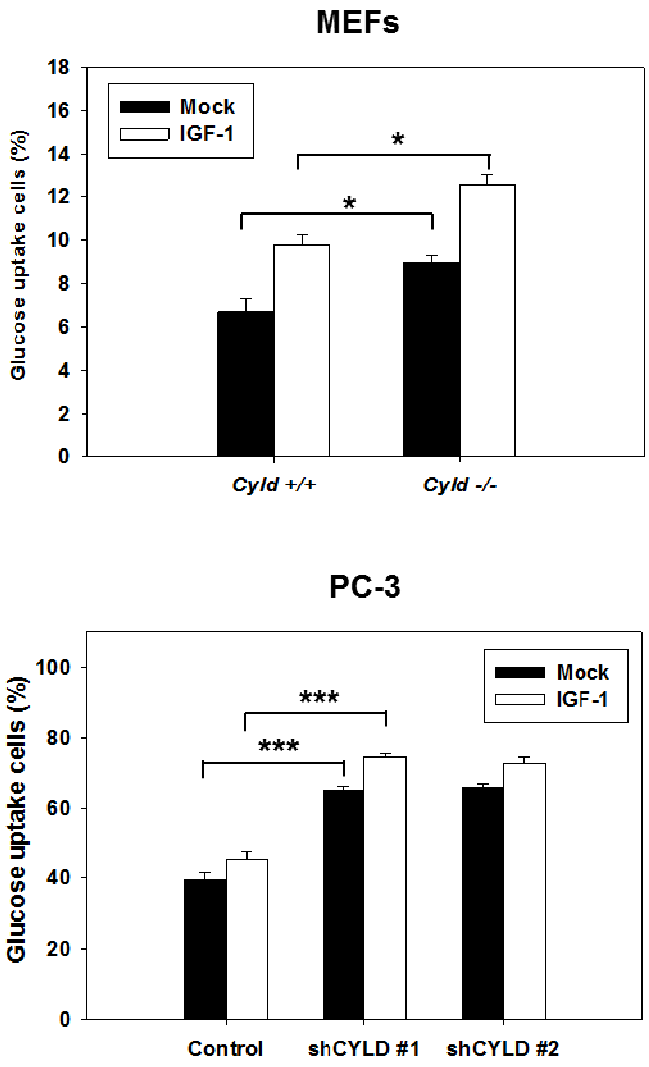

Figure 4-6. CYLD inhibits cancer cell proliferation, cell survival, and glucose uptake.

(F) Analysis of glucose uptake ratios in *Cyld*$^{+/+}$ and *Cyld*$^{-/-}$ MEFs or PC-3 cells with control or CYLD knockdown, treated with or without IGF-1 and grown in the presence of the fluorescent glucose analog 2-NBDG. Results are presented as mean percentages from three biological replicates. N.S., non-significant; ***P < 0.001 for all pairwise comparisons by Pearson $\chi 2$ test.

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4-7. CYLD serves as a tumor suppressive role in human prostate cancer development.

Loss of CYLD is found in melanoma, colon cancer, and liver cancer (151). To determine whether CYLD also plays a tumor-suppressive role in prostate cancer, we subcutaneously injected prostate cancer lines with control or CYLD knockdown into athymic nude mice and monitored tumor growth. We found that CYLD knockdown in two different prostate cancer cell lines promoted prostate tumor growth (Fig. 4-7A). We next investigated whether there was an inverse relationship between CYLD and Akt activation in human prostate cancer samples. We retrospectively analyzed representative tissue blocks of 80 primary prostate cancers in individuals who underwent radical prostatectomy. Immunohistochemistry results showed a significant negative correlation between phosphorylation of Akt at Ser^{473} and CYLD abundance (Fig. 4-7B and 4-7C). In addition, compared to early stage prostate cancer samples (stages I to IIA), advanced-stage prostate cancer samples (stages IIB to III) displayed increased phosphorylation of Akt at Ser^{473} and decreased CYLD abundance (Fig. 4-7D). These results suggest that CYLD suppresses Akt activation to inhibit prostate cancer cell growth and development. Together, our results underscore the role of CYLD in attenuating proliferation, glucose uptake, and survival of cancer cells, and thus in tumor suppression.

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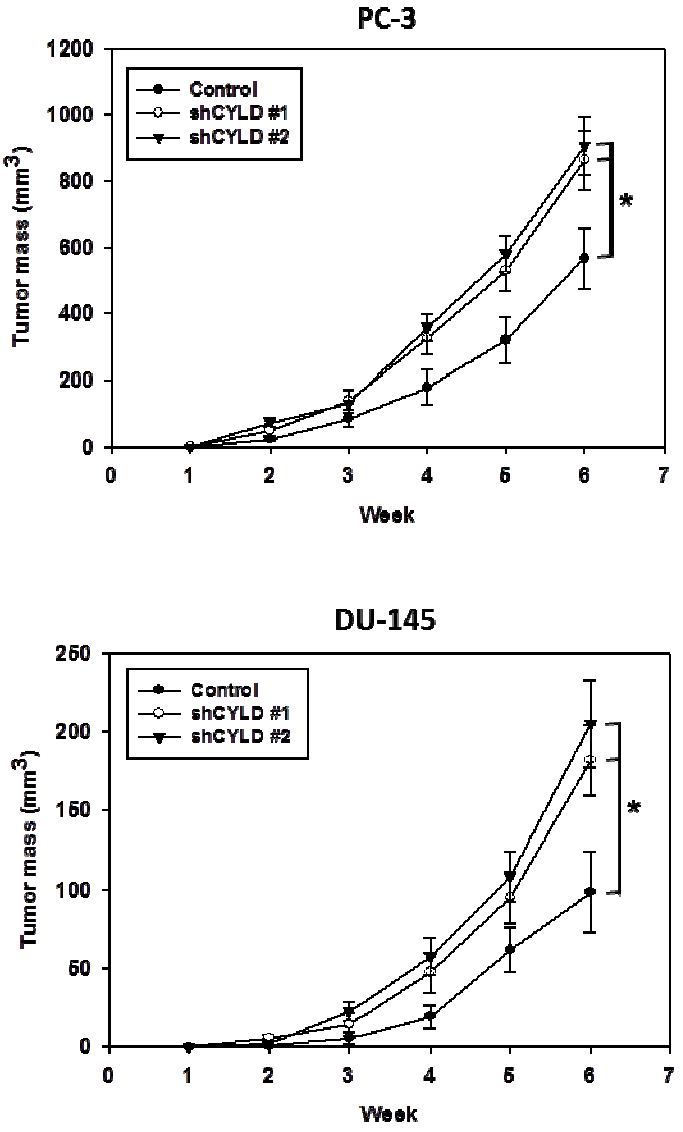

Figure 4-7. CYLD suppresses prostate cancer development.

(A) PC-3 or DU-145 cells with control or CYLD knockdown were injected into nude mice (n = 6 for each group), and the increase of tumor volume in mice was monitored every week. Results are presented as means ± SD; *P < 0.05 for all pairwise comparisons by one-way ANOVA and post hoc intergroup comparisons with Sidak test.

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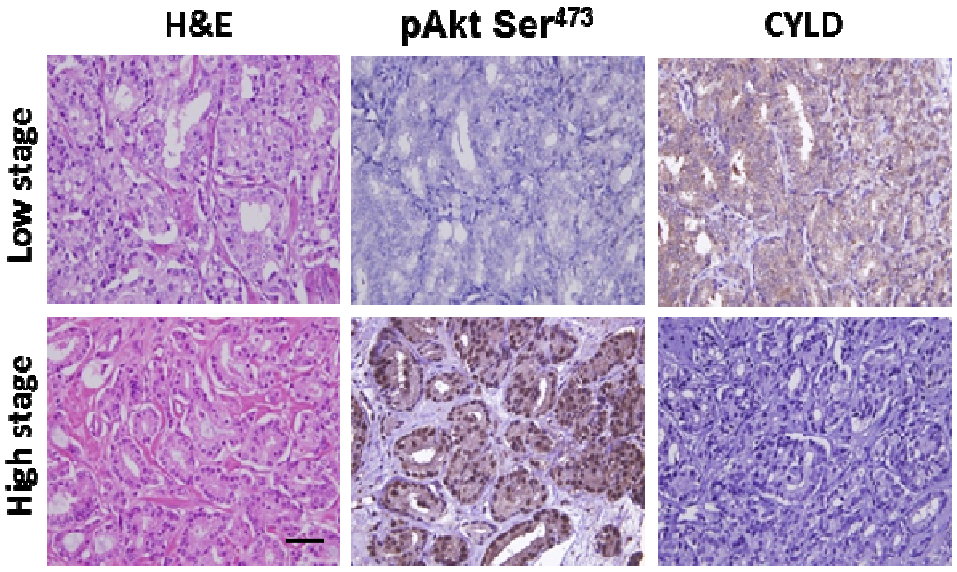

Figure 4-7. CYLD suppresses prostate cancer development.

(B) Immunohistochemistry in representative primary non-metastatic prostate cancer samples. A panel of images showing hematoxylin and eosin (H&E) staining, Akt phosphorylated at Ser^{473}, and CYLD staining in early- and advanced-stage prostate cancer samples. Scale bar, 50 mm.

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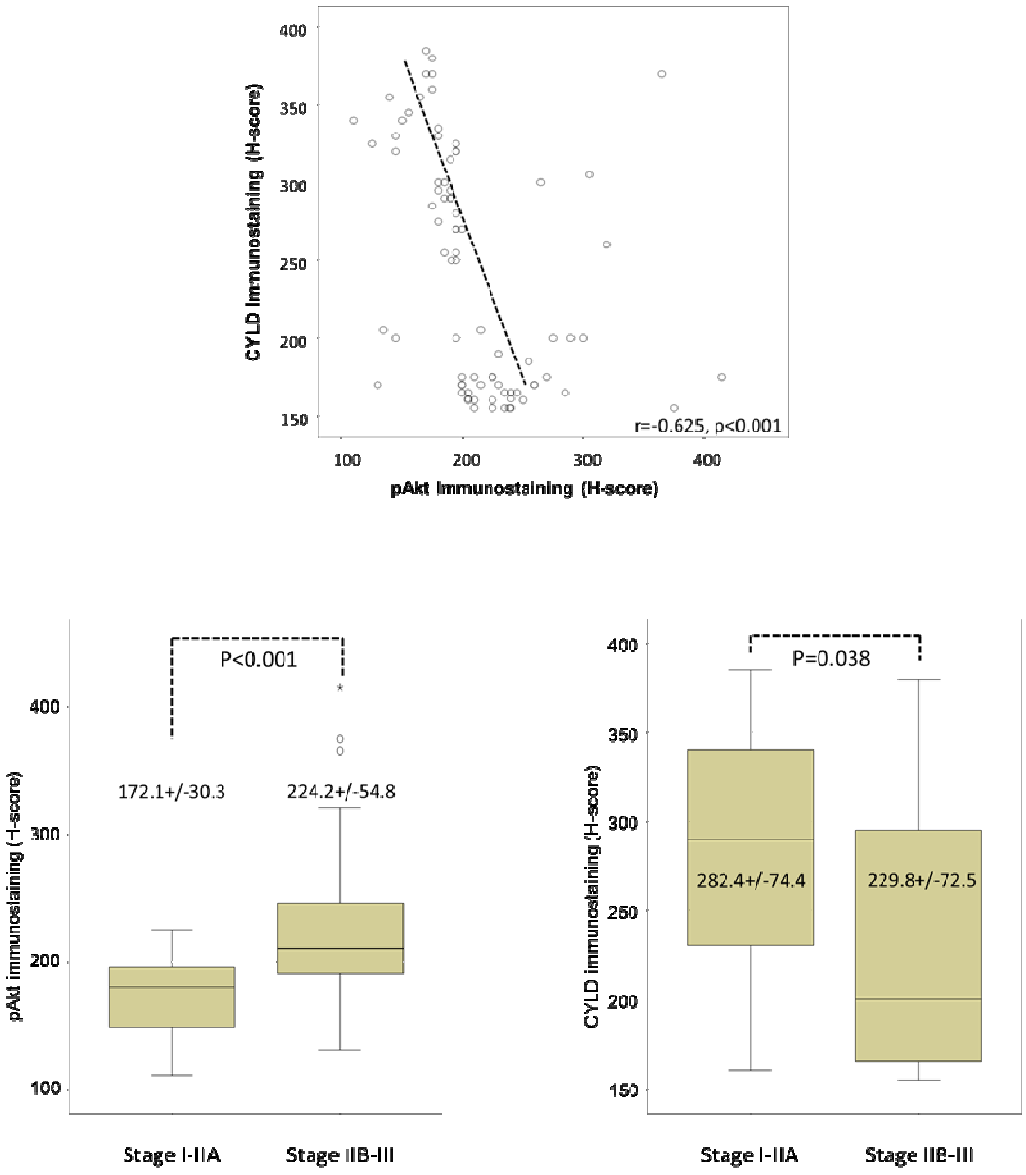

Figure 4-7. CYLD suppresses prostate cancer development.

(C) Histological score (H-score) graph showing a negative correlation between phosphorylation of Akt at Ser^{473} and CYLD abundance in prostate cancer samples. $P < 0.001$ by using Spearman's correlation and Mann-Whitney U test.

(D) Associations of H-score between phosphorylation of Akt at Ser^{473} and CYLD abundance and different tumor stages. Results are presented as means ± SD; P values were obtained with Spearman's correlation and Mann-Whitney U test.

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SUMMARY

The purpose of this study is to unveil the mechanism of ubiquitination cycle of Akt in growth factor signaling pathway and in its role in regulating activation of Akt and tumorigenesis. We have demonstrated that CYLD is a DUB for deubiquitination of Akt and it inhibits growth factor–mediated Akt signaling activation. CYLD directly removes K63-linked ubiquitination of Akt under serum-starved condition. Upon stimulation with growth factor, CYLD disassociated with Akt thereby allowing E3 ligases for Akt to promote ubiquitination and activation of Akt. In addition, CYLD deficiency promotes cancer cell proliferation, survival, glucose metabolism and human prostate cancer development. Therefore, our findings show the critical role of cycles of ubiquitination and deubiquitination of Akt in regulating membrane translocation and activation of Akt, and CYLD as a central switch for these processes.

Based on our previous findings, we propose a working model by which growth factor signaling pathways regulate ubiquitination, membrane translocation and activation of Akt (Fig. 4-8). CYLD associates with Akt and keeps Akt in a hypoubiquitinated and inactive stage by directly removing Akt K63-linked ubiquitination under serum-starved condition. However, stimulation with growth factors induce the disassociation of CYLD with Akt, and this effect allows E3 ligases for Akt to bind to Akt and ubiquitinate Akt for subsequent membrane recruitment and activation of Akt. Thus, our study suggests that CYLD serves as a critical switch to orchestrate the ubiquitination and deubiquitination cycles of Akt, thereby regulating membrane recruitment and activation of Akt, leading to tumorigenesis.

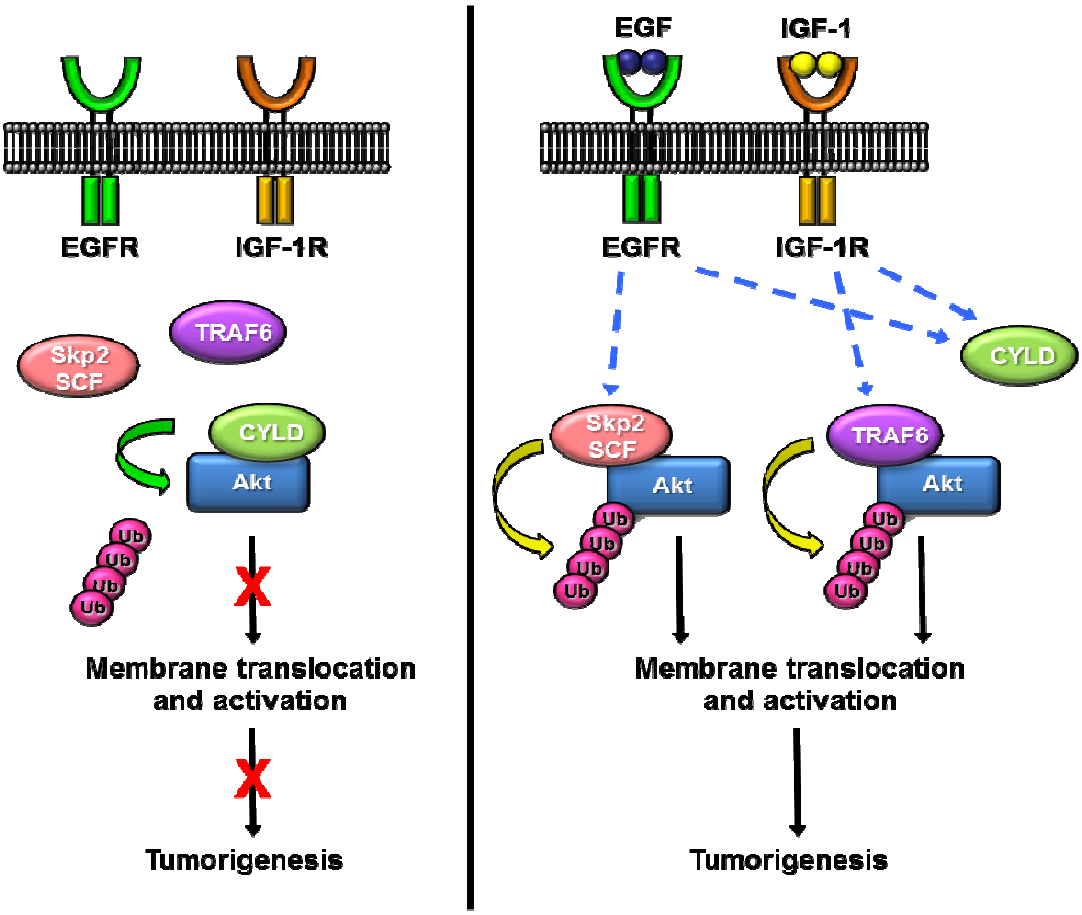

Figure 4-8. The working model for growth factor-mediated Akt ubiquitination and activation.

Under serum-starved condition of the cell (left panel), CYLD interacts with inactive Akt and keeps Akt in a hypoubiquitinated state. Upon stimulation with growth factors (right panel), growth factor signaling may promote CYLD dissociating from Akt and allow TRAF6 or Skp2 E3 ligase to interact with Akt and to elicit Akt ubiquitination, in turn facilitating Akt membrane recruitment, phosphorylation and activation. Akt activation therefore contributes to tumorigenesis by promoting cancer cells growth, survival and glucose metabolism.

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Chapter 5

Discussion

5-1. Significance

My thesis study aims to understand and unveil the long-standing mystery in the PI3K/Akt signaling field by investigating the molecular basis of growth factor signal-mediated membrane recruitment and activation of Akt. We have made several significant findings in the PI3K/Akt signaling field. We are the first to describe the molecular basis of Akt membrane recruitment by non-proteolytic ubiquitination of Akt. Furthermore, we identified TRAF6 as an E3 ligase for K63-linked ubiquitination of Akt. TRAF6 is a critical signaling modulator in TLR/IL-1R signaling pathway. However, we unexpectedly found that IGF-1 receptor signaling pathway promotes ubiquitination and activation of Akt through activating TRAF6. Also, we have established the implication of K63-linked ubiquitination of Akt in human cancer development. Thus, we proposed that TRAF6 may have potential oncogenic function in regulation of tumorigenesis. In addition, we have demonstrated the negatively regulatory mechanism for terminating ubiquitination and activation of Akt. We identified a specific DUB, CYLD, capable of influencing the activation of Akt through removing growth factor-mediated K63-linked ubiquitination of Akt. Therefore, we have established a novel tumor suppressive model of CYLD through suppressing Akt signaling, and expanded the essential tumor suppressive role of CYLD to human prostate cancer.

Aberrant activation of Akt is well-known to be a common event associated with human cancer development and progression (12, 66). However, how deregulation of Akt signaling activation leads to tumor formation is still unclear. Although developing small molecule inhibitors for targeting Akt in clinical trials are actively in progress, the results are discouraging as direct targeting of this crucial signal molecule raised serious side

effects such as diabetes and cardiac malfunctions (66). The failures of these inhibitors for Akt suggest that more thorough understanding of Akt signaling activation mechanism is required. Therefore, shedding light on the detailed mechanisms of activation and tumorigenicity of Akt not only make a significant advance in cancer research, but also provide the foundation to help the scientific community to develop innovative and effective strategies for human cancer therapy with reducing side effects.

In summary, my thesis study establishes a novel model of ubiquitination cycle of Akt in Akt signaling activation and human cancer development. This model may provide a new paradigm for researches in cell biology, cancer biology and human cancer therapy in the future.

5-2. TRAF6 mediated K63-linked ubiquitination regulates activation of Akt.

Phosphorylation of Akt has an important role in activation and oncogenic functions of Akt. In the past decade, the research involving Akt had been focused on the significance of Akt phosphorylation and how this phosphorylation was regulated. However, in the first part of my thesis study, we have demonstrated that Akt can be conjugated with non-proteolytic ubiquitination when cells were treated with known inducers for Akt activation such as growth factors or cytokines (152, 158). Ubiquitination of Akt is well correlated with phosphorylation of Akt at Thr^{308} and activation of Akt. Remarkably, this ubiquitination acts through K63-linked but not K48-linked polyubiquitination. Ubiquitination of Akt does not change stability of Akt, but it is critical for activating Akt signaling pathway (152, 158).

Following experiments in my study identified that TRAF6 is an E3 ligase for ubiquitination of Akt. *Traf6*$^{-/-}$ primary MEFs exhibited deficiencies in ubiquitination and phosphorylation of Akt in response to growth factors including IGF-1 and serum treatment (152, 158). Reconstitution of TRAF6, but not TRAF6 E3 enzyme dead mutant, restored ubiquitination and following phosphorylation of Akt in *Traf6*$^{-/-}$ MEFs, suggesting that TRAF6 E3 enzyme activity is essential for phosphorylation and activation of Akt (152, 158). The advanced mutation analysis demonstrated that ubiquitination of Akt took place at lysine 8 (K8) and lysine 14 (K14) within the PH domain of Akt, and mutations on these sites (from lysine to arginine) abolished phosphorylation and activation of Akt (152), underlining the important function of ubiquitination in activation of Akt signaling. Therefore, ubiquitination of Akt is characterized as a novel post-translational modification which has a critical role in activation of Akt.

Recent studies support our findings in PI3K/Akt signal transduction field. For example, one study demonstrated that the role of growth arrest and DNA damage-inducible a (GADD45a) in the regulation of Akt activation induced by mechanical stress. This study found that increased mechanical stress in epithelia cells promoted Akt phosphorylation through TRAF6-mediated K63-linked ubiquitination of Akt, leading to membrane translocation and activation of Akt (159). Our group also reported that another ubiquitin E3 ligase Skp2-SCF complex promoted K63-linked ubiquitination of Akt through EGFR signal transduction pathway (153), suggesting that K63-linked ubiquitination of Akt can be formed by different E3 ligases in response to distinct growth factor signaling pathways. Indeed, our notion is further corroborated by discovery of NEDD4-1 as an E3 ligase for K63-linked ubiquitination of Akt. The study found that upon stimulation with IGF-1, NEDD4-1 promoted K63-linked ubiquitination of Akt, and this type of ubiquitination subsequently enhanced nuclear translocation of Akt (160). Therefore, these studies provide more evidence to support the significance of K63-linked ubiquitination of Akt in Akt signaling pathway. We foresee that more discoveries about mechanism and/or function of K63-linked ubiquitination of Akt would be reported in the future.

5-3. Membrane translocation of Akt is regulated by ubiquitination.

Currently, it is widely perceived that Akt resides in the cytosol and moves to the plasma membrane in response to a variety of growth factor stimuli. The PH domain of Akt is clearly required for Akt membrane translocation by binding PI(3,4,5)P3 phospholipid , however, it also has a critical role in interaction with other proteins required for Akt membrane recruitment. Numerous Akt binding partners have been reported and known to regulate the activation of Akt. Some Akt binding proteins such as JNK interacting protein 1 (JIP1) and T cell leukemia-1 (TCL1) have been found to interact with the PH domain of Akt (10, 161, 162), suggesting that Akt PH domain may not only function for PI(3,4,5)P3 binding, but also serve a scaffold to recruit the crucial adaptors essential for membrane translocation and phosphorylation of Akt. This concept has been corroborated by study with ectopic expression of the PH domain of Akt. This study demonstrated that endogenous phosphorylation of Akt in cells was inhibited, whereas the PH domain mutant of Akt which lost potential to interact with other adaptor proteins but retained the PI(3,4,5)P3 binding did not (148).

Although the PH domain of Akt is well recognized to associate with PI(3,4,5)P3 for membrane translocation of Akt, it is still unknown in the initial step of how Akt translocates to the plasma membrane, where it can interact with PI(3,4,5)P3 and is subsequently phosphorylated by PDK1 and mTORC2. The current model of Akt activation proposed by the Tsichlis group indicates that before Akt translocates to the plasma membrane to bind to PI(3,4,5)P3, the inactive Akt in the cytosol may be required to associate with the essential adaptors by using its PH domain, which then promote membrane localization of Akt (148). This model has tested and supported by my thesis

study that TRAF6 promotes K63-linked ubiquitination of Akt at K8 and K14 within its PH domain, which is essential for membrane translocation and activation of Akt. (152, 158). Remarkably, ectopic expression of TRAF6 in cells stimulates polyubiquitination, membrane translocation and phosphorylation of Akt, whereas knockdown of TRAF6 protein level blocks these processes (152, 158). These results highlight the significant role of TRAF6-mediated K63-linked ubiquitination of Akt in the membrane localization and activation of Akt, suggesting the K63-linked ubiquitination has some critical regulatory roles in protein trafficking and signaling activation (93, 94).

However, TRAF6 does not appear to affect the activity of plasma membrane residing Akt. Since overexpression of TRAF6 could not further enhance phosphorylation of the myristoylated Akt mutant, which constitutively translocates to the plasma membrane (our unpublished data). TRAF6-mediated polyubiquitination of Akt does not change the capability of Akt to bind to PI(3,4,5)P3, supporting the concept that ubiquitination of Akt is unnecessary for binding to PI(3,4,5)P3 (152). Another observation in my thesis study also supported this concept, it is found that mutation of the essential ubiquitination site of Akt on the K8 residue, which is out of the PI(3,4,5)P3 binding pocket, does not change the capability of Akt to associate with PI(3,4,5)P3 (152). The results of my study suggest that both steps (K63-linked ubiquitination and PI(3,4,5)P3 interaction) are essential for membrane translocation and phosphorylation of Akt, and K63-linked ubiquitination of Akt could precede the PI(3,4,5)P3 binding on the plasma membrane (Fig. 3-10). Further studies are required for delineating the detailed mechanism of membrane translocation of ubiquitinated Akt and identifying the adaptor(s) which facilitate this process.

5-4. Hyper-ubiquitination provides hyper-activation of the cancer-associated Akt mutant

Recently, several studies reported a point mutation within the PH domain of Akt (E17K) in a variety of human cancers, including breast and colon cancer (49-53). The cancer-associated Akt E17K mutant exhibits a constitutive membrane recruitment and phosphorylation of Akt at Thr^{308}, probably caused by its enhanced PI(3,4,5)P3 binding, leading to abnormal oncogenic potential in human cancers (56). Remarkably, in my study, I have also discovered that the Akt E17K mutant exhibit enhanced K63-linked ubiquitination, and abolishment of this ubiquitination resulted in a dramatic reduction in membrane localization and phosphorylation of Akt (152, 158). These results suggest that Akt E17K mutant obtains two significant properties (enhanced ubiquitination and PI(3,4,5)P3 binding of Akt), which provide constitutive membrane localization and phosphorylation of Akt. Consequently, the results of my study suggest that targeting ubiquitination of Akt may provide potential strategies for human cancers therapy in the future.

Aside from the Akt E17K mutant, another Akt mutant (Akt E49K) was recently discovered in human bladder cancer (55). Like the Akt E17K mutant, this mutant also shows hyper-phosphorylation and activation of Akt compared with wild-type Akt (55), however the molecular basis of its hyper-activation is unknown up to now. Since the E17K mutation located at the PH domain of Akt obtains an extra lysine residue for ubiquitination, it will be exciting to examine in the future if the hyper-ubiquitination can also be detected in Akt E49K mutant and provide potential oncogenic functions of the Akt E49K mutant in human cancers.

5-5. The novel regulatory role of TRAF6 in multiple signal transduction pathways to promote activation of Akt

In addition to involvement in activation of NF-κB and p38 upon cytokines stimulation, my thesis study demonstrates that TRAF6 E3 ligase also plays an essential role for growth factor-mediated Akt signaling activation. TRAF6 promotes K63-linked ubiquitination and activation of Akt upon growth factor stimulation (152, 158). Remarkably, IGF-1 receptor (IGF-1R) signaling pathway elicits auto-ubiquitination and activation of TRAF6 corresponded with ubiquitination of Akt (152, 158). Furthermore, TRAF6 physically binds to IGF-1R and this association is interrupted upon stimulation with IGF-1 (152, 158). However, the mechanism of how IGF-1R signaling induces ubiquitination and activation of TRAF6 remains unknown. Further studies are required to investigate the molecular basis by which IGF-1R signaling promotes ubiquitination and activation of TRAF6.

From literature, we know that both endotoxin LPS and proinflammatory cytokine IL-1 promote cells survival through eliciting phosphorylation of Akt (163-165), although the detailed molecular basis by which these stimulations promote activation of Akt is currently unclear. The results of my thesis study offer the first evidence as to how LPS or IL-1 activates Akt. LPS or IL-1 stimulation acts through TLR4/IL-1R signaling-mediated TRAF6 activation. Furthermore, activated TRAF6 initiates K63-linked ubiquitination, phosphorylation and activation of Akt (152, 158). Therefore, my study suggests that TRAF6 is involved in several critical signal transduction pathways in regulating inflammation, innate immune response cell growth and survival. However, we need further studies to investigate the molecular basis of growth factor receptor signaling pathway-induced TRAF6 activity and to clarify the cross-talk between growth factor and TLR4/IL-

1R signaling pathways in the regulation of TRAF6 activity and subsequent activation of Akt.

5-6. The potential oncogenic role of TRAF6 in tumorigenesis

Even though the deregulated NF-κB signaling pathway is obviously related to human cancer development (166, 167), it is unknown whether the central mediator of this signaling pathway, TRAF6, is also engaged in tumorigenesis. Recent studies have implicated the involvement of TRAF6 in human cancer development. It is shown that TRAF6 associates with an adaptor protein p62 and their interaction is essential for ubiquitination and activation of TRAF6 (168). p62 is an oncogenic Ras responsive protein which protein level can be upregulated by Ras and is critical for Ras-mediated activation of NF-κB, cell transformation and lung cancer development (169).

Interestingly, my thesis study provides the more direct evidence regarding the potential role of TRAF6 in tumorigenesis. My study demonstrates that TRAF6 deficiency in prostate cancer cells decreases phosphorylation and activation of Akt induced by IGF-1 and diminishes tumorigenic potential of prostate cancer cells in the xenograft animal model (152). This finding extends the role of TRAF6 from toll-like receptor signaling and innate immune response to growth factor and oncogenic signaling pathways. Therefore, my study proposes that TRAF6 is a previously unidentified oncoprotein which has a critical role in human cancer development and progression. Indeed, recent studies support this notion that TRAF6 has oncogenic functions involved in several human cancers such as breast cancer, lung cancer, gastric cancer, prostate cancer and acute myeloid leukemia (170-174). Accordingly, targeting TRAF6 protein or E3 ligase activity may be a promising strategy for human cancer therapy.

5-7. The role of deubiquitinase CYLD in regulating Akt signaling pathway

The second part of my thesis study provides the insight into the mechanism of deubiquitination of Akt. We discovered CYLD as a crucial DUB which retains Akt in a hypo-ubiquitinated and inactive status. Subsequently, CYLD deficiency increases basal K63-linked ubiquitination of Akt and following membrane translocation and activation of Akt, thus enhancing oncogenic functions of Akt, such as cell proliferation, cell survival, and glucose uptake (175). Recently, Lim and colleagues (176) reported that CYLD suppresses TGF-β-induced lung fibrosis by reducing stability of Smad3 and K63-linked ubiquitination of Akt. Their study supports our finding that CYLD is a DUB for deubiquitination of Akt, and CYLD regulates biological functions of Akt through deubiquitination. However, this study did not provide any clue whether CYLD suppresses growth factor-mediated ubiquitination, membrane translocation and activation of Akt to further promote cancer development.

In my study, we discovered that CYLD binds to Akt under serum-free situation but this association is separated upon stimulation with growth factor. However, ubiquitination and phosphorylation status of Akt does not affect the association between CYLD and Akt (175). In addition, we examined that PI3K activity, which is critical for growth factor-induced activation of Akt, is unnecessary for ubiquitination of Akt elicited by growth factor and deubiquitination of Akt induced by CYLD (175). Although PI3K activity has a dispensable role in ubiquitination/deubiquitination processes of Akt, it is still essential for the phosphorylation of hyper-ubiquitinated Akt E17K mutant (175).

Recently, our group also demonstrated that another Skp2-SCF E3 ligase promotes K63-linked ubiquitination of Akt through EGFR signaling pathway (153). In addition,

another group discovered that NEDD4-1 is an E3 ligase for K63-linked ubiquitination of Akt through IGF-1R signaling pathway (160). Therefore, it is very interesting to know whether CYLD can also remove other E3 ligases-mediated K63-linked ubiquitination of Akt upon stimulation with distinct growth factor signaling pathways. Indeed, my study demonstrates that CYLD also efficiently removed Skp2-mediated ubiquitination of Akt, and CYLD deficiency cells also showed enhanced membrane recruitment and phosphorylation of Akt (175). Thus, our results support the concept that CYLD may play a universal deubiquitinase for removing K63-linked ubiquitination of Akt induced by different E3 ligases in distinct growth factor signaling pathways.

Based on the working model that I proposed in the second part of my study (Fig. 4-8), CYLD binds to Akt in the cytosol under the quiescent status to prevent the interaction of E3 ligases with Akt and also keep Akt under hypo-ubiquitinated status. Growth factor signaling triggers separation of CYLD from Akt and interaction of the E3 ligases with Akt to allow the E3 ligases-mediated ubiquitination of Akt. However, we still do not know whether the separation of CYLD from Akt results from competition binding of the E3 ligases or whether growth factor-mediated certain modification on CYLD directly leads to the disassociation of CYLD with Akt. Since this working model was established by the experiments of IGF-1 signaling-mediated association of Akt with TRAF6, we do not know whether this working model would be applicable to explain the mechanism of Skp2-mediated ubiquitination of Akt in EGFR signaling pathway. Further studies are needed to delineate the detailed mechanism of how growth factor signaling pathways regulate the disassociation of CYLD with Akt to trigger E3 ligase-mediated ubiquitination of Akt.

5-8. The potential tumor suppressive mechanism of CYLD in human cancers

My thesis study discovered for the first time the previous unrecognized role of CYLD in growth factor-induced ubiquitination, membrane translocation and activation of Akt. In addition, we demonstrate novel functions of CYLD in regulating glucose uptake, membrane localization of glucose transporter 1 (Glut1) and prostate cancer development, thus providing new exemplars for development, progression and treatment of human cancers. Although several clinical studies showed that CYLD protein level was reduced in human melanoma, liver cancer and colon cancer (151), its role in prostate cancer development remains a puzzle. In my study, we revealed that CYLD knockdown enhanced prostate cancer progression in a xenograft animal model. Furthermore, immunohistochemistry results for clinical prostate cancer patient sample exhibited that CYLD protein level was declined in advanced-stage of prostate cancer samples and reversely associated with activation of Akt (175). Therefore, our findings suggest that CYLD may serve as a tumor-suppressor in prostate cancer development by suppressing oncogenic Akt signal transduction pathway.

CYLD is also well known to negatively regulate activation of NF-κB through deubiquitination of several important mediators for NF-κB signaling activation including TRAF2, TRAF6 and NEMO (67, 122, 125-128). Since NF-κB signaling also plays an important role in human cancer development (177), it is possible that tumor suppressive functions of CYLD are not simply through regulating activation oncogenic Akt signaling. Indeed, two recent studies support this notion. A study reported that mRNA expression and protein level of CYLD were reduced dramatically in most human tumor samples including colon and liver cancers. In addition, reconstitution of CYLD expression in these tumor cell

lines markedly reduced the activity of NF-κB (178), suggesting a potential tumor suppressive role of CYLD in development of human cancers through inhibiting NF-κB signaling activation. Another study demonstrated that loss of CYLD expression resulted in constitutive NF-κB and MAPK signaling activation in hepatocytes, leading to apoptosis, inflammation and chronic liver diseases such as fibrosis and hepatocellular carcinoma (HCC) (179, 180). These results are consistent with my studies. We found that CYLD deficient prostate cancer cells treated with PI3K inhibitors impaired the increase in cell growth and survival, however the inhibitory effect was not complete, indicating that additional signaling pathways which regulate cell proliferation and survival controlled by CYLD may exist (175). Since we did not investigate the role of CYLD in activation of NF-κB signaling in my study, we cannot exclude the possibility that CYLD may negatively regulate both oncogenic Akt and NF-κB signaling pathways in human cancer development and progression. Further studies are needed to delineate how CYLD plays a decisive tumor suppressive role through inhibiting Akt and NF-κB signaling pathways in human cancers.

Chapter 6

Future Direction

In this thesis study, we demonstrated that Akt can be conjugated with K63-linked ubiquitination by growth factor signaling pathway. This modification is required for membrane recruitment and subsequent activation of Akt, and this novel regulation can be reversed by the specific deubiquitinase. Our findings provide direct evidence that ubiquitination of the critical signaling mediators downstream of growth factor receptors is also essential for protein trafficking and signal pathway activation. This significant discovery sheds new light on the PI3K/Akt signaling field, suggesting that ubiquitination of Akt may also paly similarly significant role as phosphorylation does on Akt. We foresee that the research focus on the regulation of ubiquitination of Akt and its role in human cancer development and progression represent brand-new and significant areas for further understanding of growth factor signal transduction pathways. Therefore, it is expected that more and more exciting and significant discoveries in this area will be made in the coming decade.

However, the fundamental roles of ubiquitination/deubiquitination cycles on distinct cellular functions remain unknown. The importance of this post-translational modification warrant further investigations. Answering these questions will not only allow us to better comprehend the complicity of ubiquitination cycle in Akt signaling activation, but also open a novel and exciting avenue of the role of ubiquitination in the regulation of growth factor signal transduction pathways. Here, I listed some of important questions for the future research direction:

6-1. How do growth factor signal transduction pathways regulate activity of TRAF6 E3 ligase and CYLD deubiquitinase?

In the first part of my thesis study, we have demonstrated that IGF-1R signaling pathway promotes auto-ubiquitination, and activation of TRAF6 correlated with ubiquitination of Akt (152). However, it is still unclear how IGF-1R signaling promotes ubiquitination and activation of TRAF6. My study showed that TRAF6 physically associates with IGF-1R and this interaction is disrupted by IGF-1 signaling (152). Since protein phosphorylation is well-known to regulate protein activation, it is possible that TRAF6 may undergo phosphorylation by IGF-1R tyrosine kinase upon stimulation with IGF-1. Indeed, I have demonstrated that IGF-1 treatment promoted tyrosine phosphorylation of TRAF6 (my unpublished observation). A recent study also supported this hypothesis that TLR4 signaling promotes the association of TRAF6 with Src family kinases including c-Src and Fyn. This interaction causes tyrosine phosphorylation of TRAF6 by Src family kinases and following activation of TRAF6 (181). This study suggests that IGF-1-induced activation of TRAF6 may be mediated through receptor tyrosine kinase or Src family kinases. Since another E3 ligase Skp2 had been reported previously that it can be phosphorylated by Akt and this phosphorylation promotes Skp2 E3 ligase activity (37, 38), it is also highly possible that TRAF6 may also undergo phosphorylation by EGFR family kinase or other kinases in EGFR signaling pathway.

Similar to the E3 ligases for Akt, the activity of CYLD deubiquitinase may also be regulated by growth factor signaling pathways. Based on the working model in the second part of my study, CYLD disassociates with Akt upon activation of growth factor signaling (Figure 4-8). However, how growth factor signaling induces the disassociation of CYLD

with Akt is still unknown. CYLD had been also reported previously to be phosphorylated by IKK family members and this phosphorylation inhibits CYLD enzyme activity (182, 183). These studies provide a hint that CYLD may undergo phosphorylation by growth factor receptor kinases or other kinases in growth factor signaling pathways, and this phosphorylation may either inhibit CYLD enzyme activity or induces the separation of CYLD from Akt.

6-2. What is the molecular basis of K63-linked ubiquitination in membrane recruitment and activation of Akt?

In the first part of my thesis study, we had demonstrated that TRAF6-mediated K63-linked ubiquitination of Akt upon stimulation with growth factor, and this modification is required for membrane recruitment, phosphorylation and activation of Akt (152). However, one outstanding question remains to be answered: how does the ubiquitination control membrane translocation of Akt? As the K63-linked ubiquitination has a critical function in protein/protein interaction, the K63-linked ubiquitination on the PH domain of Akt may work as a molecular scaffold to attract the crucial adaptors to promote membrane translocation and activation of Akt. To validate this hypothetical model, a comprehensive analysis towards identifying the candidates for Akt adaptor by using some systematic approaches is required. It may help us to understand how K63-linked ubiquitination controls membrane localization and activation of Akt. For example, a recent study showed that p21 protein (Cdc42/Rac)-activated kinase 1 (PAK1), a protein kinase involved in cell migration and tumorigenesis (184) associates with Akt and facilitated membrane translocation of Akt (185). How precisely PAK1 controls membrane translocation and activation of Akt is still a mystery up to now. In the future direction, it will be very interesting to investigate if ubiquitination of Akt coordinates the binding between PAK1 and Akt, then facilitating membrane translocation of Akt.

From literatures, we speculate that such adaptors for Akt are probably ubiquitin-binding proteins, which include ubiquitin-binding domains competent of interacting with mono-ubiquitin or different kinds of polyubiquitination chain (186). A variety of ubiquitin-binding domains were identified recently, including ubiquitin-associated domain,

ubiquitin-interacting motif, Npl4 zinc-finger domain, polyubiquitin-associated zinc-finger domain, GRAM-like ubiquitin binding in Eap45 domain, coupling of ubiquitin conjugation to endoplasmic reticulum degradation domain and ubiquitin conjugating enzyme variant motif (186). In addition to ubiquitination, ubiquitin-binding proteins are also discovered to be engaged in protein degradation, endocytosis of receptor, DNA damage response and protein kinase activation (186). Thus, a protein which contains such ubiquitin-binding domains may be considered as a candidate of Akt adaptor protein.

6-3. Is it feasible to develop a novel strategy by targeting ubiquitination of Akt for human cancer therapy?

As mentioned before, the activities and functions of oncogenes and tumor suppressors essential for cell growth, survival and tumorigenesis are controlled by ubiquitination and deubiquitination regulated by the E3 ligases and DUBs, respectively. Significantly, deregulation of the E3 ligases and/or DUBs is commonly discovered in various human cancers (69, 75, 187, 188). In addition, several mouse caner models have demonstrated significant roles of the E3 ligases and DUBs in cancer development and progression(69, 75, 187, 188), suggesting that E3 ligases or DUBs may be ideal drug targets for developing effective therapeutic for human cancer in the future.

Akt signaling pathway is a well-known and significant oncogenic event in cancer development. Clinical evidence support this notion that abnormal Akt activation is correlated with numerous human cancers (9, 10, 38, 189). Given the significant role of the Akt signaling in human cancers, small-molecule inhibitors which target Akt kinase activity have been developed and tested in several clinical trials (66, 190, 191). Since TRAF6 has been demonstrated as a crucial E3 ligase for ubiquitination, membrane localization and activation of Akt in my thesis study. Small molecules targeting TRAF6 E3 ligase activity may be appealing as single or adjuvant agents for human cancer therapy. Consistent with this concept, TRAF6 deficiency in prostate cancer cells decreases activation of Akt and development of prostate cancer (152). In addition, TRAF6 knockdown reduces the ability of chemotherapy resistance in prostate cancer cells (152). Thus, our findings suggest that TRAF6 targeting may be also applied with chemotherapy agents as a combinational approach to enhance the susceptibility of cancer cells.

Apart from developing small molecule inhibitors for TRAF6 targeting, the regulation of the microRNA (miR) pathways that negatively control TRAF6 protein expression may also be considered. Recent studies support this concept that miR-145 and miR-146a were demonstrated to suppress protein translation of TRAF6 and exhibited a potential tumor-suppressive function in restraining the progression of primary and metastatic breast cancer (170, 171, 192). Therefore, these results suggest that it may also be considered to manipulate miR-145 and miR-146a as a novel therapeutic strategy for human cancer therapy.

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VITA

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